ABSTRACTS SUBMITTED FOR THE JOINT MEETING OF THE SOCIETY FOR GLYCOBIOLOGY AND THE JAPANESE SOCIETY FOR CARBOHYDRATE RESEARCH

November 17–20, 2004
Honolulu, Hawaii

US/JAPAN GLYCO 2004

JOINT MEETING OF THE SOCIETY FOR GLYCOBIOLOGY AND THE JAPANESE SOCIETY OF CARBOHYDRATE RESEARCH

November 17–20, 2004 Hilton Hawaiian Village Beach Resort & Spa, Honolulu, HI

DEADLINE: OCTOBER 15

for On-line Advance Conference Registration. After October 15, please register on site.

CONFERENCE INFORMATION

Conference on Glycobiology
2019 Galisteo Street,
Building I-1,
Santa Fe, NM 87505
Tel: (505) 989-4735
Fax: (505) 989-1073
conference@glycobiology.org

CONFERENCE LOCATION

The Hilton Hawaiian Village Beach Resort & Spa offers the perfect mix of exceptional resort accommodations and classic Hawaiian hospitality. Located on Waikiki's widest stretch of beach, the Hilton Hawaiian Village features lush tropical gardens, 5 pools, waterfalls and 22 acres of oceanfront beach.

ACCOMMODATIONS

Hotel rooms for conference participants have been set-aside at the Hilton Hawaiian Village. To make a reservation, call the hotel directly, (808) 949-4321 or 1-800-HILTONS. Be sure to mention that you are attending the Joint Meeting of the Society for Glycobiology and the Japanese Society for Carbohydrate Research in order to obtain the conference rate. **Japanese attendees can make travel arrangements through a travel agent.** A list of travel agents is provided. A first night's deposit is required to confirm a room reservation, payable by check or major credit card. Any cancellations within 72 hours prior to arrival and all no-shows will be assessed a cancellation penalty equal to one night's stay. Also, there is a $50 early check-out fee. **The deadline for reservations at the conference rate is October 15, 2004.** Please make your reservations early, as the room block will sell out. The conference rates are: **$156 (Garden View), $165 (Partial Ocean View) and $174 (Ocean View).** All rooms are subject to state and local taxes (currently 11.41%).

TRANSPORTATION

The Hilton Hawaiian Village is located eight miles from Honolulu International Airport. Airport shuttles ($9-12 per person, up to an hour) and taxis (approximately $25 one way, 15-20 minutes) are available. Pairing up for taxis at the airport is recommended.

PARKING

Valet parking is available for $17 per day. Self-parking is available for $12 per day for hotel guests.

HOTEL

Hilton Hawaiian Village Beach, Resort & Spa
2005 Kalia Road, Honolulu, Hawaii 96815
Tel: (808) 949-4321or 1-800-HILTONS
www.hiltonhawaiianvillage.com

TRAVEL AGENTS FOR JAPANESE ATTENDEES

Prima Tours: contact Hitoshi Onishi at 06-6261-2011
Kintetsu Intl Express Event & Convention, Kansai: contact Yoshiji Tatsuuma at 06-6602-0224
Sanki Travel Service Co., Ltd.: contact Miki Ito at 6-6536-6801 or Tadafumi Ishizawa at 03-3947-1511

FOREIGN TRAVELERS

Please apply for your visa **at least 3 months** before the conference, to allow for processing time.

ORGANIZERS

The Society for Glycobiology
Marilynn E. Etzler (Chair and President)
Section of Molecular & Cellular Biology
University of California, Davis, CA
(530) 752-3528
president@glycobiology@org
Tamara Doering
Jeffrey D. Esko
James Paulson
Ronald Schnaar

The Japanese Society for Carbohydrate Research
Naoyuki Taniguchi (Chair)
Department of Biochemistry
Osaka University Medical School, Osaka, Japan
81-6-6879-3420
proftani@biochem.med.osaka-u.ac.jp
Sumihiro Hase
Hironobu Hashimoto (President)
Toshisuke Kawasaki
Akemi Suzuki

PROGRAM UPDATES AND OTHER USEFUL INFORMATION: ***www.glycobiology.org***

TENTATIVE PROGRAM

Additional talks will be selected from the submitted abstracts.

Wednesday, November 17

9:00 am–5:00 pm **SATELLITE SYMPOSIUM I: FROM CHEMISTRY TO SYSTEMS GLYCOBIOLOGY**
Organized by Hironobu Hashimoto, Tokyo Institute of Technology and Sumihiro Hase, Osaka University

9:00 am–5:00 pm **SATELLITE SYMPOSIUM II: PRODUCTION AND APPLICATIONS OF RECOMBINANT GLYCOPROTEINS**
Organized by Richard Cummings, University of Oklahoma and James C. Paulson, Scripps Research Institute

7:00–7:15 pm **CONFERENCE OPENING**
Opening Remarks: **Marilynn E. Etzler,** President, Society for Glycobiology
Opening Remarks: **Naoyuki Taniguchi,** Chair of Japanese organizers

7:15–8:45 pm **SESSION I: CHEMO/ENZYMATIC SYNTHESIS OF GLYCANS**
Co-Chairs: Makoto Kiso and Y.C. Lee
- **Katsumi Ajisaka**
- **Jacquelyn Gervay-Hague**
- **Yasuhiro Kajihara**
- **Monica Palcic**

8:45–10:00 pm **RECEPTION**

Thursday, November 18

8:30–10:00 am **SESSION II: GLYCAN-PROTEIN INTERACTIONS**
Co-Chairs: Richard Cummings and Reiji Kannagi
- **Linda Baum**
- **C. Fred Brewer**
- **Hiroshi Nakada**
- **Sachiko Sato**

10:00–10:30 am **BREAK AND EXHIBITS**

10:30 am–12:30 pm **SESSION III: GLYCAN PROCESSING**
Co-Chairs: Mark Lehrman and Sumihiro Hase
- **Nancy Dahms**
- **Koichi Kato**
- **Lucia Mendonca-Previato**
- **Tadashi Suzuki**
- **Tomohiko Taguchi**

12:30–2:00 pm **Lunch Break**

2:00–4:00 pm **POSTERS AND EXHIBITS**

4:00–6:00 pm **SESSION IV: GLYCOBIOLOGY OF PROKARYOTES AND NON-VERTEBRATE EUKARYOTES**
Co-Chairs: Yoshifumi Jigami and Sam Turco
- **Donald L. Jarvis**
- **Lokesh Joshi**
- **Yoshinobu Kimura**
- **Hirosi Kitagawa**
- **Debra Mohnen**

Friday, November 19

8:00–10:00 am **SESSION V: GLYCAN SIGNALING AND RECOGNITION**
Co-Chairs: Gerald Hart and Naoyuki Taniguchi
- **Jianguo Gu**
- **Jinichi Inokuchi**
- **Nobuaki Maeda**
- **Alan Rapraeger**
- **Christopher West**

10:00–10:30 am **BREAK AND EXHIBITS**

10:30 am–12:30 pm **SESSION VI: GLYCOBIOLOGY OF DEVELOPMENT**
Co-Chairs: Sen-itiroh Hakomori and Takashi Muramatsu
- **Jeffrey D. Esko**
- **Kenji Kadomatsu**
- **Shoko Nishihara**
- **Michael Pierce**
- **Scott Selleck**

12:30–2:00 pm **Lunch Break**

2:00–4:00 pm **POSTERS AND EXHIBITS**

4:00–5:00 pm **BUSINESS MEETING**

5:00–6:00 pm **KARL MEYER LECTURE**

7:00–9:30 pm **BANQUET.** Included with registration. Extra tickets for guests may be ordered.

Saturday, November 20

8:30–10:00 am **SESSION VII: GLYCOBIOLOGY AND DISEASE**
Co-Chairs: Ronald Schnaar and Akemi Suzuki
- **Tamao Endo**
- **Hudson H. Freeze**
- **Robert Sackstein**
- **Nobuhiro Yuki**

10:00–10:30 am **BREAK AND EXHIBITS**

10:30 am–12:30 pm **SESSION VIII: GLYCOMICS AND GLYCOTECHNOLOGY I**
Co-Chairs: Toshisuke Kawasaki and Vernon Reinhold
- **Anne Dell**
- **Akihiko Kameyama**
- **Kazuaki Kakehi**
- **Tsuyoshi Shirai**
- **Ajit Varki**

12:30–2:00 pm **Lunch Break**

2:00–4:00 pm **POSTERS AND EXHIBITS**

4:00–6:00 pm **SESSION IX: GLYCOMICS AND GLYCOTECHNOLOGY II**
Co-Chairs: Hironobu Hashimoto and James C. Paulson
- **Ola Blixt**
- **Jun Hirabayashi**
- **Osamu Kanie**
- **Jun Nakayama**
- **Peter H. Seeberger**

PROGRAM UPDATES AND OTHER USEFUL INFORMATION: ***www.glycobiology.org***

ADVANCE REGISTRATION FORM

DEADLINE:
OCTOBER 15
for On-line Advance Conference Registration. After October 15, please register on site.

Send to:
Conference on Glycobiology
2019 Galisteo Street,
Building I-1
Santa Fe, NM 87505 USA
Fax: (505) 989-1073
(Faxed registrations must include credit card information.)

ON-LINE REGISTRATION
Registration is available on-line at **www.glycobiology.org.** The deadline for on-line registration is October 15.

ON SITE REGISTRATION
After October 15, please register on site. On site registration fees will be $50 more than advance registration. On site registration begins at 3 pm, Wednesday, November 17 at the Hilton Hawaiian Village.

CANCELLATIONS
Notice of cancellation must be postmarked on or before November 12, 2004. A $40 processing fee will be deducted from refunds.

NAME _____ COMPANY/INSTITUTION _____

ADDRESS _____ CITY, STATE, ZIP/POSTAL CODE/COUNTRY _____

PHONE _____ FAX _____

EMAIL (RECEIPTS WILL BE E-MAILED) _____

SATELLITE SYMPOSIA 9 am–5 pm, Wednesday, November 17, $75
Check one for the optional Satellite Symposium

○ **From Chemistry to Systems Glycobiology,** organized by Hironobu Hashimoto and Sumihiro Hase
○ **Production and Applications of Recombinant Glycoproteins,** organized by Richard Cummings and James Paulson

Enter $75 for optional Satellite Symposium $ _____

CONFERENCE REGISTRATION

○ **$380,** Member of the Society for Glycobiology or Japanese Society of Carbohydrate Research (JSCR)
○ **$550,** Non-Member
○ **$175,** Student (Must be full-time in a Ph.D. program or within first two years of postdoctoral training)

Enter registration amount checked below $ _____

EXTRA BANQUET TICKETS FOR GUESTS 7 pm, Friday, November 19.
Banquet is included with registration for conference. $50 x _____ number of extra tickets

Enter extra banquet amount $ _____

ENTER TOTAL AMOUNT $ _____

○ Check is enclosed for total amount. Please make check payable to **Society for Glycobiology**
○ Charge total amount to my AMEX, VISA or MasterCard. Sorry, no other cards are accepted.

CREDIT CARD NUMBER _____ EXP DATE _____

CARD HOLDER'S NAME _____ CARD HOLDER'S SIGNATURE _____

REGISTRATION IS AVAILABLE ON-LINE AT www.glycobiology.org THROUGH OCTOBER 15.

Wednesday, November 17, 2004
7:15 – 8:45 PM
CHEMO/ENZYMATIC SYNTHESIS OF GLYCANS
Makoto Kiso and Y.C. Lee, Co-chairs

Time *Abstract Number*

7:15 PM **Chemoenzymatic Synthesis of Complex-Type Sialyloligosaccharides and Sialylglycopeptides** ; Yasuhiro Kajihara; *Graduate School of Integrated Science, Yokohama City University, 22-2, Kanazawa-ku, Yokohama, 236-0027 Japan.* 1

7:35 PM **Glycosyltransferases for the Synthesis of Oligosaccharides and Oligosaccharide Analogs**; Monica M. Palcic; *Department of Chemistry, University of Alberta, Edmonton, AB Canada T6G 2G2.*..... 2

7:55 PM **On the Regioselectivity in the Transglycosylation Using Glycosidases for the Enzymatic Syntheses of Glycans**; Katsumi Ajisaka; *265-1 Higashijima, Niitsu, Niigata 956-8603, Japan.* 3

8:15 PM **Combinatorial Chemical Approaches to Indentifying Non-natural Lectin Ligands**; Jacquelyn Gervay-Hague; *Department of Chemistry, One Shields Ave., Davis, CA 95616.*..... 4

8:35 PM **Chondroitin Polymerization is Achieved by Multiple Enzyme Complexes Consisting of Chondroitin Synthase Family Members**; Toru Uyama, Hiroshi Kitagawa, Tomomi Izumikawa and Kazuyuki Sugahara; *Department of Biochemistry, Kobe Pharmaceutical University.* 5

8:40 PM **Degree of Polymerization (DP) of Polysialic Acid (PolySia) on Neural Cell Adhesion Molecules: Development and Application of a New Strategy to Accurately Determine the DP of PolySia Chains on N-CAM**; Daisuke Nakata and Frederic A. Troy II; *Department of Biochemistry and Molecular Medicine, University of California School of Medicine, Tupper Hall, Davis, CA 95616.*..... 6

Thursday, November 18, 2004
8:30 AM – 10:00 AM
GLYCAN-PROTEIN INTERACTIONS
Richard Cummings and Reiji Kannagi, Co-chairs

Time *Abstract Number*

8:30 AM **Biological Function of Mucins Through Scavenger Receptor Present on Monocytes/Macrophages**; Hiroshi Nakada, Munetoyo Toda, Hiroaki Takagi, Tatsuro Shigenobu, Masanobu Yoshida, Takaaki Inaba, Noriko Takeuchi and Mizue Inoue; *Dept. Biotechnology, Kyoto Sangyo Univ.*..... 7

8:50 AM **Structural and Carbohydate Binding Properties of Galectins**; C. Fred Brewer; *Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY.* 8

9:10 AM **Galectin Binding to Glycoproteins and Glycans: Consequences of Low-Affinity, High-Avidity Interactions**; Linda G. Baum[1], Brianna Stillman[1], Joseph D. Hernandez[1], Alshakim Nelson[2], Jason M Belitsky[2], Daniel K Hsu[3], Fu-Tong Liu[3] and J. Fraser Stoddart[2]; *[1] Dept. of Pathology and Laboratory Medicine, UCLA School of Medicine, [2] Dept. of Chemistry, UCLA, [3] Dept. of Dermatology, UC Davis School of Medicine.* 9

9:30 AM **Galectins and Innate Immune Responses: the Soluble β-Galactoside Binding Protein Family that can Modulate Immunity Through Their Proinflammatory Cytokine-Like Activities and as Pathogen Recognition Molecules**; Sachiko Sato; *Glycobiology laboratory, Research Centre for Infectious Diseases, CHUL Research Centre, Quebec, Canada.*..... 10

9:50 AM **GlcNAc 6-*O*-Sulfotransferase (GlcNAc6ST) -1 and GlcNAc6ST–2 Regulate Lymphocyte Homing to Lymph Nodes**; Kenji Uchimura[1], Mark S. Singer[1], Durwin Tsay[1], Kenji Kadomatsu[2], Reiji Kannagi[3], Takashi Muramatsu[2] and Steven D. Rosen[1]; *[1] Department of Anatomy, Program in Immunology, University of California, San Francisco, CA [2] Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya, Japan, [3] Program of Molecular Pathology, Aichi Cancer Center, Nagoya, Japan.*..... 11

9:55 AM **H-Ras induces FucT-VII Expression via Raf and PI3K**; Dimitrios G. Zisoulis and Geoffrey S. Kansas; *Dept. of Microbiology-Immunology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Ave, Chicago, IL 60611.* 12

Thursday, November 18, 2004
10:30 AM – 12:30 PM
GLYCAN PROCESSING
Mark Lehrman and Sumihiro Hase, Co-chairs

Time *Abstract Number*

10:30 AM **Strategies for Phosphomannosyl Recognition**; Nancy M. Dahms and Jung-Ja P. Kim; *Medical College of Wisconsin, Department of Biochemistry, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226.*..... 13

10:50 AM **Endo-β-Mannosidase, A Plant Enzyme Acting on *N*-Glycan: Purification, Substrate Specificity, and Molecular Cloning**; Takeshi Ishimizu[1], Akiko Sasaki[1], Satoshi Okutani[1], Mami Maeda[1], Mai Yamagishi[1,2] and Sumihiro Hase[1]; *[1] Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan, [2] Major in Integrative Bioscience and Biomedical Enginnering, Waseda University, Okubo, Tokyo 169-8555.*..... 14

10:55 AM **NMR Structural Biology of the Intracellular Lectins that Contribute to Quality Control of Glycoproteins in Cells**; Koichi Kato.[1,2]; *[1] Nagoya City University, [2] CREST/JST.* 15

11:15 AM **Mutations in the GlcNAc-Phosphotransferase α-/β-Subunits Precursor Gene are the Molecular Basis of Both Mucolipidosis II and IIIA**; Mariko Kudo, Courtney P Kerbo and William M Canfield; *Genzyme, 800 Research Parkway, suite 200, Oklahoma City, OK 73104.*..... 16

11:20 AM **Unique *O*-glycan Biosynthesis in *Trypanosoma cruzi* Glycoproteins**; Lucia Mendonça-Previato, Adriane R. Todeschini, Wagner B. Dias, Orlando A. Agrellos, Norton Heise and José O. Previato; *Instituto de Biofísica Carlos Chagas Filho, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Cidade Universitária, Rio de Janeiro-RJ, Brasil.* 17

11:40 AM **hGPI7 Regulates the Generation of GPI-Anchors by Competing for PIG-F with PIG-O** ; Nobue Shishioh[1], Yeongjin Hong[2], Yusuke Maeda[1] and Taroh Kinoshita[1]; *[1] Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan., [2] Genomic National Center for Enteropathogenic Bacteria and Department of Microbiology, Chonnam National University Medical School, Gwangiu, S.Korea.*..... 18

11:45 AM **Cytosolic Free Oligosaccharides: Formation and Cytosolic Processing;** Tadashi Suzuki; *2-2 Yamada-oka, Suita-shi, Osaka 565-0871, Department of Biochemistry, Osaka University Graduate School of Medicine/PRESTO, JST.* 19

12:05 PM **Protein *O*-Fucosyltransferase 1(*O*-FucT-1) is a Soluble Enzyme Localized to the ER** ; Yi Luo, Kelvin B. Luther and Robert S. Haltiwanger; *Department of Biochemistry and Cell Biology, Institute of Cell and Developmental Biology, SUNY, Stony Brook, NY 11794-5215.* 20

12:10 PM **Fractionation of the Mammalian Golgi Apparatus**; Tomohiko Taguchi; *Yamadaoka 2-2, Suita, Osaka, Japan.* 21

Thursday, November 18, 2004
4:00 - 6:00 PM
GLYCOBIOLOGY OF PROKARYOTES AND NON-VERTEBRATE EUKARYOTES
Yoshifumi Jigami and Sam Turco, Co-chairs

Time *Abstract Number*

4:00 PM **Analysis and Manipulation of the Protein *N*-Glycosylation Pathway in the Baculovirus-Insect Cell System**; Donald L. Jarvis, Jared J. Aumiller, Jason R. Hollister, Ziad S. Kawar, John Pilon and Nadia Vadaie; *Department of Molecular Biology, University of Wyoming, Laramie, WY, USA.* 22

4:20 PM **Plant Sialobiology**; Lokesh Joshi, Miti Shah, Sasha Daskalova, Vinay J. Nagaraj, Chitra Prasanna, Jared Gerlach, Amy-Grace Smith, Marta Waddell, Charles R. Flynn and Linda C. Lopez; *The Biodesign Institute at Arizona State University, Tempe AZ 85287.* 23

4:40 PM **Free *N*-Glycans in Developing Plant Cells: Structural Features, Putative Function, and Related Enzymes**; Yoshinobu KIMURA; *Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University, Japan.* 24

5:00 PM **Biosynthesis and Functions of Glycosaminoglycans in *Caenorhabditis elegans***; Hiroshi Kitagawa; *Department of Biochemistry, Kobe Pharmaceutical University, Higashinada-ku, Kobe 658-8558, Japan and CREST, JST, Kawaguchi, Saitama 332-0012, Japan.* 25

5:20 PM **Biosynthesis and Function of the Plant Cell Wall Polysaccharide Pectin: Identification of a Proposed Galacturonosyltransferase Gene Family in Arabidopsis thaliana and Effect of Pectin on Human Prostate Cancer Cells**; Debra Mohnen[1], Jason D. Sterling[1], Kerry L. Hosmer[1], Melanie Atmodjo[1], Michael G. Hahn[1], V.S. Kumar Kolli[1], Crystal L. Jackson[1] and M. Vijay Kumar[2]; *[1] Complex Carbohydrate Research Center, The University of Georgia, Athens, GA 30602, [2] Medical College of Georgia and VA Medical Center, Augusta, GA 30912..* 26

5:40 PM ***Leishmania* Require Sphingolipids (Sls) for Acidocalcisome Biogenesis and Infectivity, and Salvage Sls from the Mammalian Host which are Remodeled Into Inositolphosphoceramide (IPC)**; Kai Zhang[1], Fong-Fu Hsu[2], David A. Scott[1,3], Roberto Docampo[3], John Turk[2] and Stephen M. Beverley[1]; *[1] Dept. of Molecular Microbiology, Washington University School of Medicine, St. Louis MO 63105 USA, [2] Dept. of Medicine, Washington University School of Medicine, St. Louis MO 63105 USA, [3] Dept. of Pathobiology and Center for Zoonoses Research, University of Illinois, Urbana, IL 61802.* 27

5:45 PM **Newly Discovered Neutral Glycosphingolipids in Aureobasidin A-resistant *Zygomycetes***; Kazuhiro Aoki[1], Ryosuke Uchiyama[1], Suguru Yamauchi[1], Takane Katayama[1], Saki Itonori[2], Mutsumi Sugita[2], Noriyasu Hada[3], Junko Hada[3], Tadahiro Takeda[3], Hidehiko Kumagai[1] and Kenji Yamamoto[1]; *[1] Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan, [2] Faculty of Liberal Arts and Education, Shiga University, Otsu 520-0862, Japan, [3] Kyoritsu University of Pharmacy, Minato-ku, Tokyo 105-8512, Japan.* 28

5:50 PM ***N*-linked Protein Glycosylation in *Campylobacter Jejuni***; Lorna A. Millar[1], Nicolas Cadotte[1], Luc Tessier[1], Laura Fiori[1], John Kelly[1], Harold C. Jarrell[1], Jean-Robert Brisson[1], Brenda Allan[2] and Christine M. Szymanski[1]; *[1] National Research Council of Canada, Ottawa, Canada, [2] Vaccine and Infectious Disease Organization, Saskatoon, Canada.* 29

5:55 PM **UDP-sugar Pyrophosphorylase with Broad Substrate Specificity towards Various Monosaccharide 1-phosphates from Pea Sprouts** ; Toshihisa Kotake[1], Daisuke Yamaguchi[1], Hiroshi Ohzono[1], Sachiko Hojo[1], Tomoyuki Konishi[1], Satoshi Kaneko[2], Hide-ki Ishida[3] and Yoichi Tsumuraya[1]; *[1] Department of Biochemistry and Molecular Biology, Faculty of Science, Saitama University, 255 Shimo-okubo, Sakura-ku, Saitama 338-8570, Japan, [2] Biological Function Division, National Food Research Institute, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8642, Japan, [3] the Noguchi Institute, 1-8-1 Kaga, Itabashi-ku, Tokyo 173-0003, Japan.* 30

Friday, November 19, 2004
8:00 – 10:00 AM
GLYCAN SIGNALING AND RECOGNITION
Gerald Hart and Naoyuki Taniguchi, Co-chairs

Time *Abstract Number*

8:00 AM **Roles of *N*-glycans on Integrin-mediated Signaling**; Jianguo Gu and Naoyuki Taniguchi; *Dept. Biochem., the 21st Century COE program, Osaka Univ. Med. Sch. B1, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.* 31

8:20 AM **Carbohydrate-carbohydrate Interaction-Dependent Neutrophil Chemotaxis and Phagocytosis**; Kazuhisa Iwabuchi[1,2,4], Tadashi Sato[1,3], Toshihide Kobayashi[4], Kumiko Ishii[4] and Hideoki Ogawa[1]; *[1] Institute for Environmental and Gender-specific Medicine, Juntendo University Graduate School of Medicine, [2] Laboratory of Biochemistry, Juntendo University School of Health Care and Nursing, [3] Department of Respiratory Medicine, Juntendo University Graduate School of Medicine, [4] Sphingolipid Functions Laboratory, Frontier Research System, RIKEN.* 32

8:25 AM **Significance of Cytoplasmic Prolyl Hydroxylation and Complex Glycosylation in the cellular slime mold *Dictyostelium***; Christopher M. West[1], Hanke van der Wel[1], Slim Sassi[2], Eric Gaucher[3] and Altan Ercan[1]; *[1] Dept. of Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104 USA, [2] Dept. of Anatomy & Cell Biology, University of Florida College of Medicine, Gainesville, FL, 32610 USA, [3] Foundation for Applied Molecular Evolution, Gainesville, FL 32601* 33

8:45 AM **Mapping and Quantifying O-Glycosylation Sites and Proteins Using a Mass-Spectrometry Based Comparative Proteomic Tool**; Lance Wells, Dan Sherling, Jae-Min Lim, Bryan Woosley, Ron Orlando

and Carl Bergmann; *University of Georgia, Complex Carbohydrate Research Center, Athens, GA* 34

8:50 AM **O-GlcNAc A Mediator of Cellular Function: Characterizing a Family of O-GlcNAc Binding Proteins**; Natasha E Zachara and Gerald W Hart; *Department of Biological Chemistry, The Johns Hopkins University School of Medicine.*..... 35

8:55 AM **Molecular Pathogenesis of Type 2 Diabetes Via Insulin Signaling in Membrane Microdomains: Involvement of Ganglioside GM3**; Jinichi Inokuchi; *Department of Biomembrane and Biofunctional Chemistry and 2Core Research for Evaluational Science and Technology program (CREST), Japan Science and Technology Corporation (JST), Graduate School of Pharmaceutical Sciences, Frontier Research Center for Post-Genomic Science and Technology, Hokkaido University, Kita 21-Nishi 11, Kita-ku, Sapporo 001-0021, Japan..* 36

9:15 AM **Signal Transduction of Proteoglycan-Type Protein Tyrosine Phosphatase, PTPzeta and Development of the Brain;** Nobuaki Maeda; *Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu, Tokyo 183-8526, Japan.* 37

9:35 AM **Cell Surface Localization of Heparanase on Macrophages Regulates Degradation of Extracellular Matrix Heparan Sulfate**; Norihiko Sasaki[1], Nobuaki Higashi[1], Tomohiro Taka[1], Motowo Nakajima[2] and Tatsuro Irimura[1]; *[1] Laboratory of Cancer Biology and Molecular Immunology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, [2] Tsukuba Research Institute, Novartis Pharma.*..... 38

9:40 AM **A Ligand and Carbohydrate Engagement (LACE) Assay Detects Changes in Heparan Sulfate Expression During Mouse Development**; Alan C. Rapraeger, Benjamin L. Allen, Ravishankar Ramaswamy and Xinping Yue; *Department of Pathology and Laboratory Medicine, University of Wisconsin-Madison, Madison, WI.* 39

Friday, November 19, 2004
10:30 AM- 12:30 PM
GLYCOBIOLOGY OF DEVELOPMENT
Sen-itiroh Hakomori and Takashi Muramatsu, Co-chairs

Time *Abstract Number*

10:30 AM **Deficiency of Heparan Sulfate N-Deacetylase/N-Sulfotransferase-1 in Endothelium Impairs Selectin and Chemokine Mediated Neutrophil Trafficking** ; Lianchun Wang[1], Mark M. Fuster[2], Nissi Varki[3], P. Sriramarao[4] and Jeffrey D. Esko[1]; *[1] Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, La Jolla, CA 92093, [2] Department of Medicine, University of California, La Jolla, CA 92093, [3] Department of Pathology, University of California, La Jolla, CA 92093, [4] Division of Vascular Biology, La Jolla Institute for Molecular Medicine, San Diego, CA 92121.* 40

10:50 AM ***N*-Acetylglucosamine-6-*O*-sulfotransferase-1 Deficiency Causes Loss of Keratan Sulfate in the Developing Brain and Injured Brain**; Kenji Kadomatsu[1], Haoqian Zhang[1], Kenji Uchimura[2] and Takashi Muramatsu[1]; *[1] Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan, [2] Department of Anatomy, Program in Immunology, University of California, San Francisco, CA 94143*..... 41

11:10 AM **Molecular and Cellular Functions of Proteoglycans in Tissue Assembly and Morphogenesis**; Scott B. Selleck; *Developmental Biology Center, and Depts. of Pediatrics and Genetics, Cell Biology & Development, University of Minnesota, Minneapolis, MN 55455.*..... 42

11:30 AM **Functional Glycomics Using *Drosophila* Rnai System**; Shoko Nishihara[1,2], Mika Hino[2,3], Hideki Yoshida[1,2], Norihiko Sasaki[1,2], Satoshi Goto[2,3], Hidenao Toyoda[2,4] and Ryu Ueda[2,5]; *[1] Laboratory of Cell Biology, Department of Bioinformatics, Faculty of Engineering, Soka University, [2] Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Agency (JST), [3] Genetic Networks Research Group, Mitsubishi Kagaku Institute of Life Science, [4] Department of Bio-analytical Chemistry, Graduate School of Pharmaceutical Sciences, Chiba University, [5] Invertebrate Genetics Laboratory, National Institute of Genetics.* 43

11:50 AM **Neurotic, a GDP-fucose O-fucosyltransferase, Regulates Notch Turnover and Endocytic Transportation Independently of its Enzymatic Activity;** Takeshi Sasamura[1,2], Hiroyuki O Ishikawa[3], Syunsuke Higashi[2], Maiko Kanai[1,2], Shiho Nakao[2], Nobuo Sasaki[2], Tomonori Ayukawa[2], Toshiro Aigaki[4], Katsuhisa Noda[5], Naoyuki Taniguchi[5] and Kenji Matsuno[1,2,3]; *[1] PRESTO, Japan Science and Technology Agency, [2] Department of Biological Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan, [3] Genome and Drug Research Center, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan, [4] Department of Biological Sciences, Tokyo Metropolitan University, 1-1 Minami-osawa, Hachioji-shi, Tokyo 192-0397, Japan, [5] Department of Biochemistry, Division of Molecular Therapy Science, Osaka University, Graduate School of Medicine, 1-7 Yamada-oka, Suita, Osaka 565-0871, Japan.*..... 44

11:55 AM **Action of Protein O-fucosyltransferase 1 Within the Endoplasmic Reticulum in Maturation of the Notch Receptor**; Tetsuya Okajima, Aiguo Xu, Liang Lei and Kenneth D. Irvine; *Piscataway NJ 08854.* 45

12:00 PM **Glycomic Analysis of Mouse Embryonic Stem Cell Differentiation**; Steve Dalton[1], Kelley Moremen[4], Alison Nairn[4], Gerardo Alvarez-Manilla[4], Alfred Merrill[2], Jin-Kyu Lee[4], Intaek Lee[4], Karen Abbott[4], Rick Matthews[3] and Michael Pierce[4]; *[1] Bresagen, Inc., Athens, GA 30602, [2] School of Biology, Georgia Institute of Technology, Atlanta, GA 30332, [3] Department of Neurobiology, Yale University School of Medicine, New Haven, CT 06520, [4] the Integrated Resource Center for Biomedical Glycomics, Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602.* 46

12:20 PM **Regulation of Notch Signaling by *Drosophila heparan sulfate 3-O sulfotransferase***; Keisuke Kamimura[1,2], John M. Rhodes[3], Melissa McNeely[4], Deepak Shukla[4], Koji Kimata[2], Patricia G. Spear[4,5], Nicholas W. Shworak[3] and Hiroshi Nakato[1]; *[1] Department of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN 55455, [2] Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-1195, Japan, [3] Section of Cardiology and Angiogenesis Research Center, Department of Medicine, Dartmouth Medical School, Lebanon, NH 03756, [4] Department of Microbiology-Immunology, Feinberg*

School of Medicine, Northwestern University, Chicago, IL 60611, [5] Department of Ophthalmology and Visual Sciences, University of Illinois, Chicago, IL 60612..... 47

12:25 PM **Dolichol-Cycle of Protein N-Glycosylation is Critical for Angiogenesis**; Juan A. Martinez[1], Anarda N. Gonzalez[2], Aurymar Sanchez[3], Krishna Baksi[3] and Dipak K. Banerjee[1]; *[1] Department of Biochemistry, School of Medicine, Medical Sciences Campus, University of Puerto Rico, San Juan, PR 00936-5067. USA, [2] Department of Pathology, School of Medicine, Medical Sciences Campus, University of Puerto Rico, San Juan, PR 00936-5067. USA, [3] Department of Anatomy and Cell Biology, School of Medicine, Universidad Central del Caribe, Bayamon, PR 00960-6032. USA.* 48

Saturday, November 20, 2004
8:30 - 10:00AM
GLYCOBIOLOGY AND DISEASE
Ronald Schnaar and Akemi Suzuki, Co-chairs

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8:30 AM **Protein *O*-mannosylation and its Pathology**; Tamao Endo; *Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, Itabashi, Tokyo 173-0015, Japan.* 49

8:50 AM **Expanding Perspectives on Congenital Disorders of Glycosylation**; Hudson H. Freeze, Xiaohua Wu, Ognian Bohorov, Erik Eklund and Liangwu Sun; *The Burnham Institute, 10901 N. Torrey Pines Rd. La Jolla CA*..... 50

9:10 AM **From Graft Failure to Graft-Versus-Host Disease: The Central Role of Glycans in Allogeneic Bone Marrow Transplantation**; Robert Sackstein, MD,PhD; *Harvard Institutes of Medicine, 77 Ave. Louis Pasteur, Room 671, Boston, MA, 02115.* 51

9:30 AM **Carbohydrate Mimicry between Human Ganglioside GM1 and *Campylobacter jejuni* Lipo-oligosaccharide Causes Guillain-Barre Syndrome** ; Nobuhiro Yuki; *Department of Neurology, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan.* 52

9:50 AM **A Glucose Transporter N-Glycosylation Defect Promotes Type 2 Diabetes**; Kazuaki Ohtsubo[1], Shinji Takamatsu[2], Mari T. Minowa[3], Aruto Yoshida[4], Makoto Takeuchi[4] and Jamey D. Marth[1]; *[1] Howard Hughes Medical Institute and Department of Cellular and Molecular Medicine, 9500 Gilman Drive, University of California, La Jolla, CA, 92093, [2] Biomedical Imaging Research Center, University of Fukui, 23-3 Shimoaizuki, Matsuoka, Yoshida, Fukui, 910-1193 Japan, [3] Bioinformatics Center, Hitachi, Ltd., Life Science Group, 1-3-1 Minamidai, Kawagoe, Saitama 350-1165 Japan, [4] Central Laboratories for Key Technology, Kirin Brewery Co. Ltd., 1-13-5, Fuku-ura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan.* 53

9:55 AM **Induction of Lymphocyte Homing Receptors in the Human Gastric Mucosa Infected by *Helicobacter pylori***; Motohiro Kobayashi[1,2], Junya Mitoma[1], Tsutomu Katsuyama[3], Jun Nakayama[2] and Minoru Fukuda[1]; *[1] Glycobiology Program, Cancer Research Center, The Burnham Institute, La Jolla, CA 92037 [2] Department of Pathology, Shinshu University School of Medicine, Matsumoto 390-8621, Japan, [3] Department of Laboratory Medicine, Shinshu University School of Medicine, Matsumoto 390-8621, Japan.*..... 54

Saturday, November 20, 2004
10:30 AM – 12:30 PM
GLYCOMICS AND GLYCOTECHNOLOGY I
Toshisuke Kawasaki and Vernon Reinhold, Co-chairs

Time *Abstract Number*

10:30 AM **MALDI and Electrospray MS Strategies for Glycomics and Glyco-Proteomics;** Anne Dell[1], Mark Sutton-Smith[1], David Goldberg[2], Maria Panico[1], Sara Chalabi[1], Nyet-Kui Wong[1], Paul Hitchen[1], Jihye Jang-Lee[1], Simon North[1], Victoria Ledger[1], Simon Parry[1], Stuart Haslam[1] and Howard Morris[1]; *[1] Imperial College of Science, Technology and Medicine, Department of Biological Sciences, London SW7 2AZ, UK, [2] Scripps-PARC Institute for Advanced Biomedical Sciences, Palo Alto* 55

10:50 AM **A System for Rapid Identification of Oligosaccharide on Glycoprotein Using an Observational MS^n Spectral Library**; Akihiko Kameyama[1], Norihiro Kikuchi[2], Shuuichi Nakaya[1,3], Hiromi Ito[1], Takashi Sato[1], Toshihide Shikanai[1,2], Yoriko Takahashi[2] and Hisashi Narimatsu[1]; *[1] Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan, [2] Mitsui Knowledge Industry Co., Ltd., Tokyo, Japan, [3] Shimadzu Corporation, Kyoto, Japan.*..... 56

11:10 AM **Application of Capillary Electrophoresis to Glycomics: High-Speed Screening of Carbohydrate Chains**; Kazuaki Kakehi; *Kinki University, Faculty of Pharmaceutical Sciences, Kowakae 3-4-1, Higashi-Osaka, 577-8502, Japan.*..... 57

11:30 AM **Glyco-informatics Prediction of Sugar-Protein Interactions using Protein Data Bank**; Tsuyoshi Shirai and Clara Shionyu-Mitsuyama; *Bioinformatics section, Biomolecular Engineering Reserch Institut, Furuedai 6-2-3, Suita, Osaka Japan*..... 58

11:50 AM **Histochemistry in Comparative Glycomics: Surveying the Forest Before Examining Trees**; Ajit Varki and Nissi M. Varki; *Glycobiology Research and Training Center, Departments of Pathology, Medicine and Cellular & Molecular Medicine, University of California, La Jolla, CA.*..... 59

12:10 PM **Glycan Tree Alignment and Substitution Matrix for Finding Relationships Between Glycan Linkages** ; Kiyoko F. Aoki, Minoru Kanehisa and Hiroshi Mamitsuka; *Institute for Chemical Research, Bioinformatics Center, Gokasho, Uji, Kyoto 611-0011 Japan.* 60

12:15 PM **Developing a Human Colonic Adenocarcinoma Cell Line with Unique Glycosylation Pattern as a Model System to Implement Concerted Functional Glycomics and Glycoproteomics Analysis**; Chi-Hung Lin[1], Chun-I Chen[1], Mei-Chun Yang[2], Hui-Chung Liang[1], Chia-Wei Lin[1], Sz-Wei Wu[1], Tong-Hsuan Chang[2] and Kay-Hooi Khoo[1]; *[1] Institute of Biological Chemistry, Academia Sinica, Nankang, Taipei 11529, Taiwan, [2] GlycoNex Inc., Taiwan, Hsi-Chih City, Taipei County 221, Taiwan.* 61

12:20 PM **Sample Preparation and Mass Spectral Fragmentation Methods for use in Glycobiology**; Catherine E Costello[1,2], Krystyn E Blackmun-Ross[1], Bogdan A Budnik[2], Yuri Elkin[1], Vera B Ivleva[1], Judith A Jebanathirajah[1], Jason L Pittman[1], Bo Xie[1], Mark E McComb[2], Peter B O'Connor[1,2] and Joseph Zaia[1]; *[1] Mass Spectrometry Resource, Dept of Biochemistry, Boston Univ. School of Medicine, [2] Cardiovascular*

Proteomics Center, Boston Univ. School of Medicine. 62

12:25 PM **Development of Structural Analyses of Sulfated *N*-Glycans By Mass Spectrometry And HPLC Mapping**; Hirokazu Yagi[1], Noriko Takahashi[1], Yoshiki Yamaguchi[1], Naoko Kimura[2], Reiji Kannagi[2] and Koichi Kato[1]; *[1] Graduate School of Pharmaceutical Sciences, Nagoya City University, Japan, [2] Division of Molecular Pathology, Aichi Cancer Center, Japan.* 63

Saturday, November 20, 2004
4:00 – 6:00 PM
GLYCOMICS AND GLYCOTECHNOLOGY II
Hironobu Hashimoto and James C. Paulson, Co-chairs

Time *Abstract Number*

4:00 PM **Printed Covalent Glycan Array for Ligand Profiling of Diverse Glycan Binding Proteins**; Ola Blixt[1], Steve Head[2], Tony Mondala[2], Christopher Scanlan[2], Richard Alvarez[3], Marian C. Bryan[2], Fabio Fazio[2], Daniel Calarese[2], James Stevens[2], Nahid Razi[1], Irma van Die[4], Dennis Burton[2], Ian A, Wilson[2], Richard Cummings[3], Margaret E. Huflejt[5], Nicolai Bovin[6], Chi-Huey Wong[2] and James C. Paulson[2]; *[1] Glycan Synthesis and Protein Expression Core D, Consortium for Functional Glycomics, Department of Molecular Biology, CB216, The Scripps Research Institute, 10550 North Torrey Pines Road, 92037, La Jolla,CA [2] The Scripps Research Institute, [3] University of Oklahoma Health Science Cntr., Biochemistry & Molecular Biology, Oklahoma City, [4] Department of Molecular Cell Biology & Immunology, VU medical center, Amsterdam, the Netherlands, [5] Sidney Kimmel Cancer Center, San Diego, [6] Shemyakin & Ovchinnikov Institute, Russian Academy of Sciences, Moscow.* 64

4:20 PM **Glycan Profiling by Means of Lectins**; Jun Hirabayashi; *Research Center for Glycoscience, AIST Tsukuba Central 6, 1-1-1, Higashi, Tsukuba, Ibaraki 305-8566, Japan.* 65

4:40 PM **Synthesis and Utilization of Trisaccharide Library in Sequencing of Oligosaccharide;** Osamu Kanie; *Mitsubishi Kagaku Institute of Life Sciences (MITILS) Machida-shi, Tokyo 194-8511 Japan.* 66

5:00 PM **A Subtype of Human Gastric Mucins Expressing Alpha1,4-Glcnac-Capped *O*-Glycans Functions as a Natural Antibiotic Against *Helicobacter Pylori* Infection**; Jun Nakayama[1], Masatomo Kawakubo[1], Yuki Ito[1], Motohiro Kobayashi[1,3], Michiko N Fukuda[3], Minoru Fukuda[3] and Tsutomu Katsuyama[2]; *[1] Department of Pathology, Shinshu University School of Medicine, [2] Department of Laboratory Medicine, Shinshu University School of Medicine, [3] Glycobiology Program, Cancer Research Center, The Burnham Institute.* 67

5:20 PM **Carbohydrate Microarrays as Versatile Tools for Glycobiology**; Peter H. Seeberger and Matthew D. Disney; *ETH Zürich, Laboratory for ORganic Chemistry, HCI F315, Wolfgang-Pauli-Str. 10, 8093 Zurich, Switzerland .* 68

5:40 PM **Profiling Glycosylation using Azidosugars in vivo**; Danielle H Dube, Jennifer A Prescher, Chi N Quang and Carolyn R Bertozzi; *Department of Chemistry, University of California, Berkeley, Berkeley, CA 94720.* 69

5:45 PM **Carbohydrate-based Cancer Vaccines**; Yanbin Pan[1], Peter Chefalo[2], Nancy Nagy[2], Clifford Harding[2] and Zhongwu Guo[1]; *[1] Department of Chemistry, Case Western Reserve University, 10900 Cleveland, OH 44106, [2] Department of Pathology, Case Western Reserve University, 10900 Cleveland, OH 44106, USA.* 70

5:50 PM **Oligosaccharide-MTX Conjugate for the Analyses of UGGT Mediated Glucosylation**; Kiichiro Totani[1], Yoshito Ihara[2], Ichiro Matsuo[1,2] and Yukishige Ito[1,2]; *[1] RIKEN (The Institute of Physical and Chemical Research), Wako, Saitama 351-0198 Japan Saitama 351-0198, Japan , [2] Nagasaki University School of Medicine, Nagasaki 852-8523 Japan, [3] CREST (JST), Kawaguchi 322-1102 Japan.* 71

5:55 PM **Characterization of Cargo Receptors ERGIC-53, VIP36, and VIPL Using Monoclonal Antibodies**; Norihito Kawasaki, Naoki Matsumoto and Kazuo Yamamoto; *Dept. of Integrated Bioscience, Grad. School of Frontier Sciences, Univ. of Tokyo.* 72

POSTER SESSION 1
Thursday November 18, 2004
2:00 – 4:00 PM
Topics: Chemo/Enzymatic Synthesis of Glycans, Glycan Processing, Glycan-Protein Interactions

Chemo/Enzymatic Synthesis of Glycans

Poster Number *Abstract Number*

1 **Chondroitin Polymerization is Achieved by Multiple Enzyme Complexes Consisting of Chondroitin Synthase Family Members**; Toru Uyama, Hiroshi Kitagawa, Tomomi Izumikawa and Kazuyuki Sugahara; *Department of Biochemistry, Kobe Pharmaceutical University.* 5

2 **Degree of Polymerization (DP) of Polysialic Acid (PolySia) on Neural Cell Adhesion Molecules: Development and Application of a New Strategy to Accurately Determine the DP of PolySia Chains on N-CAM**; Daisuke Nakata and Frederic A. Troy II; *Department of Biochemistry and Molecular Medicine, University of California School of Medicine, Tupper Hall, Davis, CA 95616.* 6

3 **Engineering of a Thioligase: Randomized Mutagenesis of the Acid/Base Residue Leads to the Identification of Improved Catalysts;** Johannes Müllegger[1], Michael Jahn[1], Hong-Ming Chen[1], R. Antony J. Warren[2] and Stephen G. Withers[1]; *[1] Protein Engineering Center of Excellence, Department of Chemistry, University of British Columbia, Vancouver, BC V6T 1Z1, Canada , [2] Protein Engineering Center of Excellence, Department of Microbiology, University of British Columbia, Vancouver, BC V6T 1Z1, Canada* . . 73

4 **Solid-phase Synthesis of the Glycopeptide Bearing Consecutive N- and O-glycans**; Yuko Nakahara, Hironobu Hojo and Yoshiaki Nakahara; *Department of Applied Biochemistry, Institute of Glycotechnology, Tokai University,.* 74

5 **A Practical Synthetic Approach Towards Core 2 O-Glycan-Linked Glycopeptides**; Yutaka Takano, Hironobu Hojo, Naoya Kojima and Yoshiaki Nakahara; *Department of Applied Biochemistry, Institute of Glycotechnology, Tokai University, Kitakaname 1117, Hiratsuka, Kanagawa, 259-1292, Japan .* 75

6 **Synthesis of Bisubstrate Type Inhibitor of *N*-Acetylglucosaminyltransferases Using Polymer-Resin Hybrid Strategy**; Shinya Hanashima[1], Shino Manabe[1,3], Kei-ichiro Inamori[2], Naoyuki Taniguchi[2] and Yukishige Ito[1,4]; *[1] RIKEN (The Institute of Physical and Chemical*

25 **Toward Synthesis of Complex type *N*-glycans of Helminth Origin**; Jun Nakano[1,2], Hiromichi Ohta[2] and Yukishige Ito[1,3]; *[1] RIKEN (The Institute of Physical and Chemical Research), [2] Keio University, [3] CREST (JST).* 95

26 **Synthesis of New Polyester from Biomass** ; Kohsaku Okuyama, Daisuke Yoshida and Kenichi Hatanaka; *1Institute of Industrial Science, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8050 Japan* 96

27 **Synthesis of Asparagine-linked Glycan Chains: Toward Understanding Glycoprotein Quality Control**; Ichiro Matsuo, Shinya Hagihara, Kiichiro Totani, Toshinori Kashiwagi and Yukishige Ito; *The Institute of Physical and Chemical Reseach (RIKEN) and CREST Project, JST.*..... 97

28 **Di-*tert*-butylsilylene(DTBS) Group-Directed α-Selective Galactosylation: Effect of Leaving Groups of Glycosyl Donor;** Akiyoshi Kimura[1], Akihiro Imamura[1,2], Hideharu Ishida[1] and Makoto Kiso[1,2]; *[1] Department of Applied Bioorganic Chemistry, Gifu University, 1-1 yanagido,Gifu 501-1193, Japan, [2] CREST, Japan Science and Technology Agency (JST), 1-1 Yanagido, Gifu 501-1193, Japan.* 98

29 **Characterization and Utilization of a β-primeverosidase-like *endo*-β-glycosidase from *Penicillium Multicolor***; Satoshi Amarume[2], Takeomi Murata[1], Toshitaka Tsuruhami[2], Shigeharu Mori[2], Jun Hiratake[3], Kanzo Sakata[3] and Taichi Usui[1]; *[1] Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan, [2] Amano Enzyme Inc. Gifu R&D Center, 4-179-35 Sue-cho Kakamigahara, Gifu 509-0108, Japan, [3] Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan.* 99

30 **Enzymatic Synthesis of Chondroitin by Chondroitin Polymerase from *Escherichia Coli* Strain K4**; Nobuo Sugiura[1,2], Hideo Mochizuki[1,2], Hiroshi Maeda[2] and Koji Kimata[1]; *[1] Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-1195, Japan, [2] Central Research Laboratories, Seikagaku Corporation, Tateno, Higashiyamato, Tokyo 207-0021, Japan.* 100

31 **Characteristics of an α 2,6-sialyltransferase Produced by a Marine Bacterium, *Photobacterium Damsela* JT0160**; Takeshi Yamamoto, Yoshimitsu Takakura and Hiroshi Tsukamoto; *Plant Innovation Center, Japan Tobacco Inc., Higashibara, Iwata, Shizuoka 438-0802, Japan.* 101

32 **Facile Formation of a Macrocyclic Structure of Tricolorin a Using Intramolecular Glycosidation**; Kazuteru Kaneko, Natsuko Yanagiya, Tetsuya Furuike and Nobuo Sakairi; *Graduate School of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan.* 102

33 **Protein Modification by C-Glycosylation: Synthesis of C-Mannosyl Tryptophan and Its Relative Peptides**; Shino Manabe[1,2] and Yukishige Ito[1,2]; *[1] RIKEN, Hirosawa, Wako-shi, Saitama, 351-0198, Japan, [2] CREST, JST, Honcho, Kawaguchi, Saitama, 332-0012, Japan.* 103

34 **Synthesis of Novel Macromolecular Compounds Containing Cyclodextrin**; Kiyoshi Tamura, Naoko Yoshie and Kenichi Hatanaka; *Institution of Industrial Science, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan.* 104

35 **Production and Immobilization of Human Glycosyltransferases att the Yeast Cell Surface Through Fusion with Cell Wall Pir Proteins;** Yoh-ichi Shimma, Fumie Saitou, Fumi Oosawa and Yoshifumi Jigami; *Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology. AIST central 6, Tsukuba, Ibaraki, 305-8566, Japan..* 105

36 **Synthetic Study on HPG Series Gangliosides, New Species Isolated from the Sea Cucumber; Construction of Fucosyl α(1→4)/α(1→8) Sialic Acid** ; Yukari Katano[1], Hiroyo Shimizu[1], Yusuke Koike[1], Sachiko Koizumi[1], Hiromune Ando[2], Hideharu Ishida[1] and Makoto Kiso[1]; *[1] Department of Applied Bioorganic Chemistry, Gifu University,1-1 Yanagido, Gifu, Gifu, Japan 501-1193, [2] Life Science Research Center, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan.* 106

37 **Facile Synthesis of the 3-*O*-Alkyl-D-Glucose Derivatives and their Antifungal and Fruiting-Inducing Activities Against A Basidiomycete**; Takeshi Nishimura, Yumi Magae and Seiji Ohara; *Department of Wood Improvement, Forestry and Forest Products Research Institute, PO Box 16, Tsukuba Norin Kenkyu Danchi-nai, Ibaraki 305-8687, Japan.* 107

38 **Facile Procedure to Introduce ω-Mercaptoalkyl Residue into Glucose and Sialyl α (2→3)Lactose as a Versatile Spacer for Further Conjugations**; Yuki Kato[1], Mikio Ishihara[1,2], Hideharu Ishida[1] and Makoto Kiso[1]; *[1] Department of Applied Bioorganic Chemistry, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan, [2] Tokyo Kasei Kogyo, 4-10-2 Nihonbashihonmachi Tyuouku Tokyo 103-0023, Japan.*..... 108

39 **Synthesis of the Dibranched Core OS Expressed in Neisserial LOS Using a Versatile Hep Intermediate**; Kazuyuki Ishii, Kiyoka Toyota, Tsuyoshi Ichiyanagi and Ryohei Yamasaki; *Department of Biochemistry and Biotechnology, Tottori University, Tottori, Japan.*..... 109

40 **Design and Synthesis of Bacterial Ceramidase Inhibitors**; Masamitsu Miyamori[1], Masanori Yamaguchi[1], Hideharu Ishida[1], Aki Ogura[2], Hatsumi Mondyusyo[2], Makoto Ito[2] and Makoto Kiso[1]; *[1] Department of Applied Bioorganic Chemistry, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan, [2] Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan.* 110

41 **Highly α-specific 2-*O*-acylation of KDO using Akyl and Aryl Isocyanates**; Tsuyoshi Ichiyanagi and Ryohei Yamasaki; *Department of Biochemistry and Biotechnology, Tottori University, Tottori, Japan.*..... 111

42 **In vitro O-glycosylation of E. coli-produced Therapeutic Proteins Using Recombinant Glycosyltransferases**; Shawn Defrees[1], Zhi-Guang Wang[1], Arthur E. Scott[1], Jin Wang[1], Ruye Xing[1], David Zopf[1], Dominique L. Gouty[1], Eric R. Sjoberg[1], Krishnasamy Panneerselvam[1], Els C.M. Brinkman-Van der Linden[1], Robert J. Bayer[1], Mads A. Tarp[2] and Henrik Clausen[2]; *[1] Neose Technologies, Inc. Horsham, PA and San Diego, CA, [2] Faculty of Health Sciences, School of Dentistry, Copenhagen, Denmark.* 112

43 **Chemoenzymatic Synthesis of CMP-Sialic Acid Analogs and Sialates Using Microbial Enzymes** ; Xi Chen, Hai Yu, Hui Yu and Rebekah Karpel; *Department*

Poster Session 1 Topic
Glycan-Protein Interactions

75 **Recombinant α-dystroglycan Produced in CHO Cells is Heavily Glycosylated Independent of β-Dystroglycan Expression, and Binds to Recombinant Agrin Domains In A Ca++- and Sialylation-Independent Fashion**; Qiang Qu and Frances Smith; *University of Massachusetts Medical School, Shriver Center for Mental Retardation, 200 Trapelo Road, Waltham, MA, 02452..* 143

76 **Pancreatic Proteinases Show Novel Carbohydrate-Binding Activities and Bind to Glycoreceptors in the Intestinal Brush Border Membrane**; Hiroko Takekawa, Chieko Ina, Yuki Ihara and Haruko Ogawa; *Graduate School of Humanities and Sciences, Ochanomizu University, Tokyo 112-8610 Japan.* 144

77 **Utility of Pseudoproteoglycan (Pseudopg) Probes that Simulate PG Supramolecular Structure**; Keiko Sato, Makiko Yamagami and Haruko Ogawa; *Graduate School of Humanities and Sciences, Ochanomizu University, Tokyo 112-8610 Japan.* 145

78 **Cross-linking Studies of Concanavalin A and Dioclea Grandiflora Lectin with Synthetic Multivalent Carbohydrates**; Tarun K. Dam[1], Stefan Oscarson[2], Rene Roy[3], Frank Macaluso[1] and C. Fred Brewer[1]; *[1] Albert Einstein College of Medicine, Bronx, New York, [2] Stockholm University, Stockholm, [3] Universite du Quebec a Montreal, Canada.* 146

79 **Use of Synthetic Tetrasialosides for Modeling of Multivalent Interactions between Influenza Virus and Its Cellular Receptors**; Alexander Chinarev[1], Alexander Tuzikov[1], Alexander Sobol[1], Anne Imberty[2], Alexandra Gambaryan[3] and Nicolai Bovin[1]; *[1] Shemyakin&Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia, [2] CERMAV-CNRS, Grenoble, France, [3] Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow, Russia.* 147

80 **Thermodynamic Analysis for the Sugar Recognition of Fbs1**; Shinya Hagihara, Kiichiro Totani, Ichiro Matsuo and Yukishige Ito; *RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan, CREST, JST Kawaguchi 332-1102, Japan .* 148

81 **Design of the Blood Group Ab Glycotope**; Elena Korchagina[1], Tatiana Pochechueva[1], Polina Obukhova[1], Anne Imberty[2], Robert Rieben[3] and Nicolai Bovin[1]; *[1] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russian Federation, [2] CERMAV-CNRS (affiliated with Université Joseph Fourier), BP 53, F-38041 Grenoble Cedex 09, France, [3] Cardiology, Swiss Cardiovascular Center Bern, University Hospital, CH-3010 Bern, Switzerland.* 149

82 **The Effects on the Expression of Human Immune Molecules on Human Pmbcs by N-Glycosylation Inhibitors**; Xiao-Lian Zhang[1], Juan-juan Wu[1] and Xiang Zhou[2]; *[1] Department of Immunology, College of Medicine, Wuhan University, Wuhan 430071, P R China, [2] College of Chemistry and Molecular Science, Wuhan University, Wuhan 430072, P R China.* 150

83 **Characterization of CD28 Glycosylation in T cells**; Mary A. Turner and Dorothy E. Lewis; *Department of Immunology, One Baylor Plaza, Baylor College of Medicine, Houston, TX 77030.* 151

84 **Binding of Verotoxin 1 to Globotriaosyl Ceramide-Cholesterol Lipid Rafts Is Determined by Fatty Acid Content**; Radhia Mahfoud, Murugesapillai Mylvaganam, Beth Binnington Boyd and Clifford Lingwood; *Research Institute, Division of Infection, Immunity, Injury and Repair, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada.* 152

85 **Endoglycan is Expressed on Leukocyte Subsets with Modifications that Suggest Dual Functions as a Selectin Ligand and Chemokine Presentation Molecule;** Sheena C. Kerr, Claudia B. Fieger and Steven D. Rosen; *Department of Anatomy, Program in Immunology, University of California, San Francisco, California, 94143-0452.* 153

86 **Isoforms of Glucosidase II Differently Recognize Both Oligosaccharides and Protein Moieties of High-Mannose Bearing Glycoproteins**; Masahiko Ikekita and Masaaki Toyoda; *Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba, Japan.* 154

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Friday November 19, 2004
2:00 – 4:00 PM

Topics: Glycobiology of Prokaryotes and Non-Vertebrate Eukaryotes
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51 **Lectins in Hemocytes of the Eastern Oyster, *Crassostrea Virginica*: Biochemical and Molecular Characterization;** Keiko Saito[1], Satoshi Tasumi[1], Matthew J. Jenny[2], M. Theresa Loreto[1], Gregory W. Warr[2] and Gerardo R. Vasta[1]; *[1] Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD [2] Marine Biomedicine and Environmental Sciences, Medical University of South Carolina, Charleston, SC.* 267

52 **Properties of Glycan Oligomers From Developing Cotton Fibers**; Allen K. Murray[1], Robert L. Nichols[2], Robert J. Seward[3] and Catherine E. Costello[3]; *[1] Glycozyme, Inc., 17935 Sky Park Circle, Ste. E, Irvine, CA 92614 , [2] Cotton Incorporated, 6399 Weston Parkway, Cary, NC 27513, [3] Mass Spectrometry Resource, Boston University School of Medicine, Boston, MA 02118.* 268

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System Whereas Msiglec-F is Restricted to Eosinophils; Jiquan Q. Zhang[1], Björn Biedermann[2], Lars Nitschke[2] and Paul R. Crocker[1]; *[1] Division of Cell Biology and Immunology, The Wellcome Trust Biocentre at Dundee, University of Dundee, Dundee, GB.[2] Institute of Virology and Immunobiology, University of Würzburg, Würzburg, Germany.* 283

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73 **The Mouse Na^+/K^+-Atpase β1-Subunit Contains a K^+-Dependent Cell Adhesion Activity to β-Glcnac-Terminating Glycans**; Kiyoshi Furukawa[1], Masahiko Ikekita[2], Takeshi Sato[1], Yoshihiro Akimoto[3], Hayato Kawakami[3] and Noriaki Kitamura[1,2]; *[1] Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, [2] Faculty of Science and Technology, Tokyo University of Science, [3] Department of Anatomy, Kyorin University School of Medicine.* 285

74 **Developmental Regulation of Lipid Raft Signaling in the Rat Cerebellum**; Naoko Suzuki[1,2], Kohei Yuyama[1,2], Yutaka Sanai[1] and Kohji Kasahara[1,2]; *[1] Tokyo Metropol. Inst. Med. Sci., [2] PRESTO, JST.* 286

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80 **Oligochitin Elicitor Receptor in Plasma Membrane of Suspension-Cultured Rice Cells – Solubilization, Purification and Molecular Cloning,** Hanae Kaku[1], Yuki Ito[1], Yoko Nishizawa[1], Naoshi Dohmae[2], Koji Takio[3], Naoko Ishii-Minami[1], Ayako Katou[1], Eiichi Minami[1] and Naoto Shibuya[1,4]; *[1] NIAS, Tsukuba, 305-8602, Japan, [2] RIKEN, Wako, 351-0198, Japan, [3] RIKEN, Mikatsuki-town, 679-5148, Japan, [4] Meiji University, Kawasaki, 214-8571, Japan* 292

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82 **Characterization of Inhibitory Signaling Motifs of Natural Killer Cell Receptor, Siglec-7: Attenuated Recruitment of Phosphatases by the Receptor is Attributed to Two Amino Acids in the Motifs**; Toshiyuki Yamaji, Takane Teranishi and Yasuhiro Hashimoto; *Glyco-chain Functions Laboratory, RIKEN Frontier Research System, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan* 294

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84 **Glyco-receptor for Gangliosides and Galnac-Terminated Oligosaccharides to Induce Cdc42-Mediated Neuronal Development**; Hideyoshi Higashi[1,2] and Yoshio Hirabayashi[1]; *[1] Neuronal Circuit Mechanisms Research Gr., Brain Science Institute, RIKEN, Wako, Saitama, Japan, [2] CREST, JST, Kawaguchi, Saitama, Japan.* 296

85 **Membrane Redistribution of Gangliosides and GPI-Anchored Proteins under Conditions of Lipid Raft Isolation** ; Marija Heffer-Lauc[1], Gordan Lauc[2], Leonardo Nimrichter[3], Susan E. Fromholt[3] and Ronald L. Schnaar[3]; *[1] University of Osijek School of Medicine, Department of Biology, J. Huttlera 4, 31000 Osijek, Croatia, [2] University of Osijek School of Medicine, Department of Chemistry and Biochemistry, J. Huttlera 4, 31000 Osijek, Croatia, [3] Departments of Pharmacology and Neurosciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.* 297

86 **EGF-induced Mobilization of Neu3 Sialidase to Membrane Ruffles in A431 Cells**; Kazunori

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Genome and Drug Research Center, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan, [4] Department of Biological Sciences, Tokyo Metropolitan University, 1-1 Minami-osawa, Hachioji-shi, Tokyo 192-0397, Japan, [5] Department of Biochemistry, Division of Molecular Therapy Science, Osaka University, Graduate School of Medicine, 1-7 Yamada-oka, Suita, Osaka 565-0871, Japan. 44

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104 **Loss of Polysialic Acid Results in Serious Neurological Defects in Mice**; Birgit Weinhold[1], Herbert Hildebrandt[2], Ralph Seidenfaden[2], Martina Mühlenhoff[1], Melanie Oschlies[1] and Rita Gerardy-Schahn[1]; *[1] Abteilung Zelluläre Chemie, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, 30625 Hannover, Germany, [2] Institut für Zoologie, Universität Hohenheim, Garbenstr. 30, 70593 Stuttgart, Germany.* 312

105 **Identification of Glycoproteins with Embryoglycan in Embryonal Carcinoma Cells**; Hisako Muramatsu[1], Yoshihiro Oda[1] and Takashi Muramatsu[1,2]; *[1] Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya, Japan, [2] Department of Health Science, Faculty of Psychological and Physical Sciences, Aichi Gakuin University, Nisshin, Aichi, Japan.* 313

106 **Expression of a Secreted Form of Dally, a *Drosophila* Glypican, Induces Overgrowth Phenotype by Affecting Action Range of Hedgehog**; Hiroshi Nakato[1], Satomi Takeo[2], Cyndy Firkus[1] and Takuya Akiyama[1]; *[1] Department of Genetics, Cell Biology and Development, The University of Minnesota, Minneapolis, MN 55455, USA, [2] Department of Biology, Tokyo Metropolitan University, Hachioji-shi, Tokyo 192-0397, Japan.* 314

107 **Absence of Polysialic Acid Affect Neural Development in ST8Sia II and ST8Sia IV Double Knockout Mice;** Kiyohiko Angata[1], Jamey D Marth[2] and Minoru Fukuda[1]; *[1] Glycobiology Program, The Burnham Institute, La Jolla, CA 92037, [2] Howard Hughes Medical Institute, University of California San Diego, La Jolla, CA 92093.* 315

108 **Differentiation of F9 Embryonal Carcinoma Cells Requires Up-regulation of Gangliosides Biosynthesis and Formation of Membrane Microdomains**; Takashige Sato[1], Asif Mohammad Zakaria[1], Satoshi Uemura[1], Atsushi Ishii[3], Yoshiko Ohno-Iwashita[4], Yasuyuki Igarashi[1] and Jin-ichi Inokuchi[1,2]; *[1] Department of Biomembrane and Biofunctional Chemistry, Hokkaido University, [2] Core Research for Evaluational Science and Technology program (CREST), Japan Science and Technology Corporation (JST), Graduate School of Pharmaceutical Sciences, Frontier Research Center for Post-Genomic Science and Technology, [3] Division of Project Research, Creative Research Initiative "Sousei" Hokkaido University, [4] Biomembrane Research Group, Tokyo Metropolitan Institute of Gerontology.* 316

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110 **Neuronal Expression of the Chondroitin Sulfate Proteoglycans RPTPβ and Phosphacan**; Noriko Hayashi[1], Seiji Miyata[1], Mayumi Yamada[1], Kaeko Kamei[1] and Atsuhiko Oohira[2,3]; *[1] Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan, [2] Department of Perinatology and Neuroglycoscience, Institute for Developmental Research, Aichi Prefectural Colony, Kasugai, Aichi 480-0392, Japan, [3] Graduate School of Medicine, Nagoya University, Nagoya 466-8550, Japan.* 318

111 **Significance of Glycosphingolipid Synthesis in Zebrafish Embryogenesis**; Mitsuyoshi Masuda[1], Noriyuki Sueyoshi[1], Sayaka Satoh[1], Yukihiro Yoshimua[1], Keishi Sakaguchi[1], Nozomu Okino[1], Yoshio Hirabayashi[2] and Makoto Ito[1]; *[1] Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka, Japan, [2] RIKEN, Saitama, Japan.* 319

112 **Gene Expression Profiling of Mouse Postnatal Cerebellar Development Using Oligonucleotide Microarrays Designed to Detect Differences in Glycoconjugate Expression**; Frances I. Smith[1], Qiang Qu[1], Tim Gilmartin[2] and Steven Head[2]; *[1] University of Massachusetts Medical School, Shriver Center, 200 Trapelo Road, Waltham. MA. 02452, [2] The Scripps Research Institute, DNA Array Core Facility, 10550 N Torrey Pines Rd, La Jolla CA, 92037.* 320

113 **Small Changes in Immune Cell Development and Function as a Result of Tissue Specific Disruption of Heparan Sulfate**; Omai B. Garner[1], Vibeke Videm[2] and Jeffrey D. Esko[1]; *[1] Department of Cellular Molecular Medicine, Glycobiology Research and Training Program, University of California, San Diego, La Jolla, California, 92093-0687, [2] Department of Immunology and*

Baltimore, MD, [2] Hopital Sainte-Justine and Departement de Pediatrie, University of Montreal, Montreal, Quebec, [3] Division of Infectious Diseases, Department of Medicine, University of Maryland Medical System, Baltimore, MD. 351

144 **Study of the Role of Sulfate Modification of Heparan Sulfate in Vivo and Screening for Compounds that Modify it.** ; Sally E Stringer[1], Melissa A Rusch[2], Eleanor Y Chen[2], Stephen C Ekker[2] and Scott B Selleck[2,3]; *[1] University of Manchester Department of Medicine, Manchester Royal Infirmary, Oxford Road, Manchester. M13 9WL. UK, [2] Department of Genetics, Cell Biology and Development, University of Minnesota, 6-160 Jackson Hall, 321 Church St SE, Mineapolis. MN 55455. USA, [3] Department of Pediatrics, University of Minnesota, Medical School. MMC 391, 420 Delaware St SE, Minneapolis. MN 55455* 352

145 **Comparative Analysis of Oligosaccharides in Human Milk and Feces using High Performance Thin Layer Chromatography**; David S. Newburg[1], Josef J. Polak III[1], Susan N. Wilhelm[2], Mary Beth Stepans[2], Mary S. Jacewicz[3] and Deborah S. Newburg[1]; *[1] Program in Glycobiology, Mucosal Immunology Laboratory, Massachusetts General Hospital, Boston, MA 02129, [2] University of Nebraska College of Nursing, Scottsbluff, NE 69361, [3] Shriver Center of the University of Massachusetts Medical School, Waltham, MA 02452.* 353

POSTER SESSION 3
Saturday November 20
2:00 – 4:00 PM

Topics: Glycobiology and Disease, Glycomics and Glycotechnology I, Glycomics and Glycotechnology II

Glycobiology and Disease

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1 **A Glucose Transporter N-Glycosylation Defect Promotes Type 2 Diabetes**; Kazuaki Ohtsubo[1], Shinji Takamatsu[2], Mari T. Minowa[3], Aruto Yoshida[4], Makoto Takeuchi[4] and Jamey D. Marth[1]; *[1] Howard Hughes Medical Institute and Department of Cellular and Molecular Medicine, 9500 Gilman Drive, University of California San Diego, La Jolla, California, 92093, [2] Biomedical Imaging Research Center, University of Fukui, 23-3 Shimoaizuki, Matsuoka, Yoshida, Fukui, 910-1193 Japan, [3] Bioinformatics Center, Hitachi, Ltd., Life Science Group, 1-3-1 Minamidai, Kawagoe, Saitama 350-1165 Japan, [4] Central Laboratories for Key Technology, Kirin Brewery Co. Ltd., 1-13-5, Fuku-ura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan.* 53

2 **Induction of Lymphocyte Homing Receptors in the Human Gastric Mucosa Infected by *Helicobacter pylori***; Motohiro Kobayashi[1,2], Junya Mitoma[1], Tsutomu Katsuyama[3], Jun Nakayama[2] and Minoru Fukuda[1]; *[1] Glycobiology Program, Cancer Research Center, The Burnham Institute, La Jolla, CA 92037, USA, [2] Department of Pathology, Shinshu University School of Medicine, Matsumoto 390-8621, Japan, [3] Department of Laboratory Medicine, Shinshu University School of Medicine, Matsumoto 390-8621, Japan.* 54

3 **Characteristics of I β6-N-acetylglucosaminyltransferase-deficient Mice** ; Guo-Yun Chen[1], Hisako Muramatsu[1], Mineo Kondo[2], Nobuyuki Kurosawa[1], Yozo Miyake[2], Naoki Takeda[3] and Takashi Muramatsu[1,4]; *[1] Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya Japan, [2] Department of Ophthalmology, Nagoya University School of Medicine, Nagoya, Japan, [3] Center for Animal Resources and Development, Kumamoto University, Kumamoto, Japan, [4] Department of Health Science, Faculty of Psychological and Physical Sciences, Aichi Gakuin University, Nisshin, Aichi, Japan.* 354

4 **Effects of Immunomodulatory Drugs on Galectin-3**; Sanja Dabeliæ, Jerka Dumiæ and Mirna Flögel; *Department of Biochemistry and Molecular Biology, Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovaèiæa 1, 10000 Zagreb, Croatia.*..... 355

5 **Molecular Interaction Between Fetuin and 15 Phenolic Compouds, Including Environmental Hormones by SPR Analysis.** ; Naoko Masuda, Yasuhiro Ozeki and Shinji Asakura; *Facul. of Sci, Dept.of Biochem. Yokohama City Univ. 22-2,Seto, Kanazawaku,Yokohama Japan 236-0027.* 356

6 **TNF Alpha-Induced Insulin Resistance In Adipocytes As A Membrane Microdomain Disorder:Involvement of Ganglioside Gm3**; Kazuya Kabayama[1,2], Takashige Sato[1], Futoshi Kitamura[1], Satoshi Uemura[1], Byoung Won Kang[1], Yasuyuki Igarashi[1] and Jin-ichi Inokuchi[1,2]; *[1] 1Department of Biomembrane and Biofunctional Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, [2] Core Research for Evaluational Science and Technology program (CREST), Japan Science and Technology Corporation (JST).* 357

7 **Molecular Mechanism and Evolutional Analysis of Human Influenza a Virus N2 Neuraminidase Genes Based on the Transition of the Low-Ph Stability of Sialidase Activity**; Tadanobu Takahashi[1], Takashi Suzuki[1], Takehiko Saito[2], Chao-Tan Guo[1,3], Kazuya I.-P. Jwa Hidari[1], Daisei Miyamoto[1] and Yasuo Suzuki[1]; *[1] Department of Biochemistry, University of Shizuoka, School of Pharmaceutical Sciences, CREST, JST, and COE Program in the 21st century, Shizuoka 422-8526, Japan, [2] Department of Virology III, National Institute of Infectious Diseases, Musashi-Murayama 208-0011, Tokyo, Japan, [3] Institute of Bioengineering, Zhejiang Academy of Medical Sciences, 182 Tianmushan Road, Hang Zhou 310013, China.* 358

8 **Beta-Glucan "Pro-Drugs": Large Beta-Glucans are Processed by Macrophages that Secrete a Bioactive Fragment that Primes Neutrophils to Kill Antibody-Targeted Tumor Cells**; Gordon D. Ross[1], Daniel J. Allendorf[1], Richard D. Hansen[1], Jun Yan[1] and Brian K. Brandley[2]; *[1] Tumor Immunobiology Program, James Graham Brown Cancer Center, University of Louisville, Louisville, KY, [2] Pharmaceutical Division, Biopolymer Engineering, Inc., Eagan, MN.* 359

9 **The bioavailability and Pharmacokinetics of Chondrosine after Oral and Intravenous Single Dose Administration in Mice**; Shuichi Kusano[1], Naoko Igarashi[2], Shinobu Sakai[2], Hidenao Toyoda[2] And Toshihiko Toida[2]; *[1] The Research Institute Of Fuji Sangyo Co. Ltd., Marugame, Kagawa, Japan, [2] Graduate School Of Pharmaceutical Sciences, Chiba University, Chiba, Japan.* 360

10 **Endogenously Produced Ganglioside GM3 Endows Anticancer Drug Resistance Phenotype by Upregulating Bcl-2 Expression in 3LL Lewis Lung Carcinoma Cells**; Mariko Noguchi[1], Kazuya

Kabayama[1,2], Satoshi Uemura[1], Byoung Won Kang[1], Masaki Saito[3], Yasuyuki Igarashi[1] and Jin-ichi inokuchi[1,2]; *[1] Department of Biomenbrane and Biofunctional Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, [2] Core research for evaluational science and technology program (CREST), Japan science and technology corporation (JST), [3] Pharmacodynamics, Meiji Pharmaceutical University.* 361

11 **Increased Fucosylation and Branching of Serum Tranferrin N-Glycans in Long-Term Untreated Galactosemic Patients.** ; Luisa Sturiale[1], Rita Barone[2], Giovanni Sorge[2], Marco Zaffanello[3], Agata Fiumara[2], Giuseppe Impallomeni[1] and Domenico Garozzo[1]; *[1] Istituto di Chimica e Tecnologia dei Polimeri -CNR - Viale R. Margherita 6, 95123 Catania, Italy, [2] Centro per le Malattie Metaboliche Ereditarie - Dipartimento di Pediatria - Università di Catania - Via S. Sofia 78, 95125 Catania, Italy, [3] Dipartimento di Pediatria - Università di Verona - Piazzale L. Scuro, 37134 Verona, Italy....* 362

12 **Carbohydrate Phenotyping of Human and Animal Milk Glycoproteins: the Use of Pig Milk as an Inhibitor of Helicobacter Pylori Colonization**; Anki Gustafsson[1,5], Anna Hultberg[5], Rolf Sjöström[3], Imre Kacskovics[4], Michael E Breimer[2], Thomas Borén[3], Lennart Hammarström[5] and Jan Holgersson[5]; *[1] Department of Clinical Chemistry, Sahlgrenska University Hospital, Göteborg, [2] Department of Surgery, Sahlgrenska University Hospital, Göteborg, [3] Department of Medical Biochemistry and Biophysics, Umeå University, Umeå, [4] Department of Physiology and Biochemistry, Faculty of Veterinary Science, Szent István University, Budapest, [5] Division of Clinical Immunology, Karolinska Institutet, Karolinska University Hospital at Huddinge, Stockholm.* 363

13 **GM3-binding Peptides Inhibit Influenza Virus Infection**; Teruhiko Matsubara[1], Machiko Sumi[1], Takao Taki[2] and Toshinori Sato[1]; *[1] Department of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan, [2] Molecular Medical Science Institute, Otsuka Pharmaceutical Co. Ltd., 463-10 Kagasuno, Kawauchi, Tokushima 771-0192, Japan.* 364

14 **Directed Evolution of Sugar-Replica Peptide Having Hemagglutinin-Binding Ability**; Ai Onishi, Teruhiko Matsubara and Toshinori Sato; *Department of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan.* 365

15 **4-methylumbelliferon Inhibits Metastasis of Melanoma Cells;** Atsushi Kon[1], Shuichi Yoshihara[2], Daisuke Kudo[1], Ikuko Kakizaki[1], Ryousuke Taniguchi[1], Masahiko Endo[1] and Keiichi Takagaki[1]; *[1] Department of Biochemistry, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan, [2] Department of Surgery, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan.* 366

16 **GlcNAc 6-*O*-sulfotransferase-2 Ectopically Expressed in Ovarian Mucinous and Clear Cell Carcinoma Cells**; Akira Kanoh[1], Akira Seko[1,2], Masaru Sakamoto[3] and Katsuko Yamashita[1]; *[1] Dept. of Biochem., Sasaki Institute, [2] CREST, [3] Kyoundo Hospital.* 367

17 **Differeces in Receptor Specificity of Human and Murine Respiroviruses**; Takashi Suzuki[1], Toru Takimoto[2], Allen Portner[3], Yoko Akashi[1], Tomomi Suzuki[1], Chika Hosokawa[1], Hiroo Ueyama[1], Tadanobu Takahashi[1], Chao-Tan Guo[1], Kazuya I.-P. Jwa Hidari[1], Daisei Miyamoto[1] and Yasuo Suzuki[1]; *[1] University of Shizuoka School of Pharmaceutical Sciences, Department of Biochemistry, CREST JST, and COE Program in the 21st Century, Shizuoka, Japan, [2] University of Rochester, Medical Center, Department of Microbiology and Immunology, Rochester, United States, [3] St. Jude Children's Research Hospital, Division of Virology Department of Infectious Diseases, Memphis, United States.* 368

18 **The Glycosylation of Alpha-1-Acid Glycoprotein In Feline Infectious Peritonitis**; Kerry Cunningham[1], P.David Eckersall[1], Diane.D Addie[1] and Kevin.D Smith[2]; *[1] Institute of Comparitive Medicine, Faculty of Veterinary Medicine, University of Glasgow, Glasgow, Scotland, [2] Department of Bioscience, University of Strathclyde, Glasgow, Scotland.* 369

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20 **Identification of an Endothelial Cell Surface Receptor Involved in Sialyl-Lewisx-Dependent Cancer Metastasis to the Lung;** Michiko N. Fukuda, Hiroto Kawashima and Minoru Fukuda; *Glycobiology Program, Cancer Research Center, The Burnham Institute, La Jolla, California* 371

21 **Increase of GQ1b Gangliosides in the Adult Mouse Hippocampus Following Kindled-Seizures;** Keiko Kato[1], Masao Iwamori[2] and Yoshio Hirabayashi[3]; *[1] Department of Laboratory Animal Medicine, Division of Veterinary Science, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka, 599-8531, Japan, [2] Department of Biochemistry, Faculty of Science and Technology, Kinki University, 3-4-1, Kowakae, Higashi-osaka, Osaka, 577-8502, Japan, [3] Neuronal Circuit Mechanisms Research Group, Brain Science Institute, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama, 351-0198, Japan.* 372

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24 **Apoptosis of Human Leukemia Cells Induced by β-Xylooligosaccharides from Green Alga**; Kuniko Yamaguchi[1], Kana Sumoto[1], Noriyuki Sueyoshi[1], Yoshiya Izumi[2], Makoto Ito[1] and Takashi Nakamura[1]; *[1] Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka, Japan, [2] Advanced Technology Research Laboratory, Oji Paper Co., Ltd., Tokyo, Japan.* 375

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57 **Dangerous Chicken H5 Influenza Viruses Recognize Sulfated Receptor, Neu5Acα2-3Galβ1-4(6-HSO_3)GlcNAc**; Nicolai Bovin[1], Alexandra Gambaryan[2], Alexander Tuzikov[1] and Galina Pazynina[1]; *[1] Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry RAS, 117997, ul. Miklukho-Maklaya, 16/10, Moscow, Russia, [2] Chumakov Institute of Poliomyelitis and Viral Encephalitides Moscow, 142782, Russia.*... 408

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60 **Comparison of Ion Exchange/Immunoassay with Immunoaffinity - Mass Spectrometry in Determination of Underglycosylated Transferrin in Alcohol Dependent Patients;** John F. O'Brien[1], Jean M. Lacey[1], Daniel K. Hall-Flavin[2] and Gabrielle J. Melin[2]; *[1] Department of Laboratory Medicine & Pathology, [2] Department of Psychiatry, Mayo Clinic, Rochester, MN 55905.* 411

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62 **Respiratory Syncytial Virus (RSV) G Glycoprotein-Induced Eosinophilia Requires IL-5, Eotaxin, and CD4+ T Cells: Illness is Dependent on CD4+ T Cells and Not Eosinophils** ; Teresa R. Johnson and Barney S. Graham; *40 Convent Drive MSC 3017, Building 40 Room 2504, Bethesda, MD 20892-3017.* 413

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89 **Recombinant GNE Alters the Expression of Proteins Important for Muscle Functions and Provides Insights into the Cellular Basis of HIBM**; Hao Chen[1], Stephan Hinderlich[2] and Kevin J Yarema[1]; *[1] Department of Biomedical Engineering,Johns Hopkins University,Baltimore MD 21218,USA, [2] Charite-Universitatsmedizin Berlin, Campus Benjamin Franklin,Institut fur Biochemie und Molekularbiologie,Arnimallee 22,14195 Berlin-Dahlem,Germany.* 440

90 **Feedback Control Mechanisms, Not the Potential Catalytic Epimerization Activity of GNE, Determines Bulk Metabolic Flux through the Sialic Acid Biosynthetic Pathway** ; Zhiyun Wang[1], Zhonghui Sun[1], Hao Chen[1], Stephan Hinderlich[2] and Kevin J Yarema [1]; *[1] Whitaker Biomedical Engineering Institute, The Johns Hopkins University, 3400 N.CharlesSt, Baltimore MD 21218 , [2] Charite - Universitatsmedizin Berlin, Campus Benjamin Franklin, Institut fur Biochemie und Molekularbiologie, Arnimallee 22, 14195 Berlin-Dahlem, Germany.* 441

91 **The Interaction of Galectin-1 with Mast Cells Promotes Surface Exposure of Phosphatidylserine without Inducing Apoptosis or Degranulation;** Daniel R Callejon[1], Marlise AB Montes[1], Lilian Cataldi[1], Rodrigo Orlandini-Castro[2], Maria C Jamur[2], Constance Oliver[2], Sean R Stowell[3], Richard D Cummings[3] and Marcelo Dias-Baruffi[1]; *[1] Faculdade de Ciencias Farmaceuticas de Ribeirao Preto (DACTB) - Universidade de Sao Paulo - Brasil, [2] Dept of Molecular Biology and Cell Biology - Faculty of Medicine of Ribeirao Preto - University of Sao Paulo - Brazil, [3] Dept of Biochemistry and Molecular Biology - University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104.* 442

92 **Detection of Galectin-1 and Anti-galectin-1 Antibodies in Serum and Cerebrospinal Fluid derived from Lupus Patients;** Renata Delalibera-Joviliano[1], Juliana S Oliveira[1], Marlise AB Montes[1], José C Rosa[2], Lewis J Greene[2], Eduardo A Donadi[3], Richard D Cummings[4] and Marcelo Dias-Baruffi[1]; *[1] Faculdade de Ciencias Farmaceuticas de Ribeirao Preto (DACTB) - Universidade de Sao Paulo-Brasil, [2] Dept of Molecular Biology and Cell Biology and Protein Chemistry Center - Faculty of Medicine of Ribeirao Preto - University of Sao Paulo - Brazil, [3] Faculty of Medicine of Ribeirao Preto - University of Sao Paulo - Brazil, [4] Depto of Biochemistry and Molecular Biology - University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104.* 443

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Poster Session 3 Topic
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117 **Design and Functional Evaluation of Photoaffinity Labeling Reagents Having Fluorescent Glycopeptides**; Suzuki Masaya, Ohguro Yuuya, Nishida Yoshihiro and Kobayashi Kazukiyo; *Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan.* 464

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122 **Development of a Quantitative Real-time PCR Method for Analyzing Transcript Abundance of Glycan-Related Genes in Mouse Tissues and Embryonic Stem Cells**; Alison V. Nairn and Kelley W. Moremen; *The Complex Carbohydrate Research Center at the University of Georgia, Athens, GA 30602.* 469

123 **Optimization of TLC/VC MALDI-FTMS Method for Ganglioside Analysis;** Vera B Ivleva, Bogdan A Budnik, Peter B O'Connor and Catherine E Costello; *Boston University School of Medicine, Boston, MA.* 470

124 **Multiple Sampling in Single Cell Analysis: α-Glucosidase II Assays and Beyond**; Glen K Shoemaker, Leon H Lau, Justin Lorieau and Monica M Palcic; *Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2G2.* 471

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(1) **Chemoenzymatic Synthesis of Complex-Type Sialyloligosaccharides and Sialylglycopeptides**

Yasuhiro Kajihara.
Graduate School of Integrated Science, Yokohama City University, 22-2, Kanazawa-ku, Yokohama, 236-0027 Japan.

Complex-type oligosaccharides play an important role on proteins. However, the oligosaccharides show a structural heterogeneity so called "glycoform". This heterogeneity is a hindrance in the investigation of an oligosaccharide function. In order to gain insight into the function of oligosaccharides on a protein, glycoprotein having a pure oligosaccharide should be synthesized. For this synthetic approach, a convenient preparation method of several oligosaccharides and a synthetic method of glycoprotein are essential. It is known that complex-type dibranch-α-2,6-sialyloligosaccharide can be prepared from egg yolk on a gram scale. Our synthetic method of diverse complex-type oligosaccharides adopts branch-specific glycosidase digestion (β-galactosidase, N-acetyl-β-glucosaminidase, and α-mannosidase) toward asialobranch, which was prepared from the complex-type dibranch-disialyloligosaccharide by a partial acid treatment. In addition, we used α-2,3-sialyltransferase in order to obtain complex-type 2,3-sialyloligosaccharides. These oligosaccharides thus obtained were used for the synthesis of sialyloligopeptides by solid phase peptidesynthesis. The sialyl residues on the peptide were also substituted by NeuAc analogues by use of a CMP-NeuAc analogue and sialyltransferase in order to manipulate bioactivity of the sialylglycopeptide. These modifications of NeuAc would be useful to find a candidate for therapeutic peptide drugs, since sialyloligosaccharide is expected to prolong the life-time of peptide in blood. This presentation will describe in detail our recent progress toward chemoenzymatic synthesis of diverse complex-type oligosaccharides and sialylglycopeptides.

(2) **Glycosyltransferases for the Synthesis of Oligosaccharides and Oligosaccharide Analogs**

Monica M. Palcic.
Department of Chemistry, University of Alberta, Edmonton, AB Canada T6G 2G2.

Oligosaccharides and oligosaccharide analogs have broad applications as biochemical probes. They are used as inhibitors of carbohydrate-protein binding, as enzyme substrates, as tags to direct targeting, as ligands for affinity chromatography, as bioanalytical standards, and for receptor identification or localization. The target oligosaccharides are usually between two and five sugar residues in size. These molecules can be chemically synthesized, but this can require many months for the first of a new class of structure or for the preparation of analogs. Also the methods of organic synthesis are often too harsh to be applicable to the modification of isolated glycoproteins, glycolipids or cells. Biocatalytic approaches employing enzymes (glycosyltransferases, glycosidases, glycosynthases) or engineered whole cells are increasingly used for the selective formation of glycosidic linkages. We are developing methods for using glycosyltransferases, both isolated and cloned, for the synthesis of the oligosaccharides and their analogs. Glycosyltransferase reactions are stereospecific and regiospecific occurring with the transfer of a monosaccharide from a nucleotide donor to acceptor in a single step without the need for substrate protection and deprotection. Biomimetic oligosaccharide synthesis of natural sequences requires access to the correct enzymes and their corresponding sugar nucleotides, along with defined reactions conditions and methods for reaction monitoring. Synthesis of oligosaccharide analogs requires the glycosyltransferases to cross-react with unnatural substrates, either donor or acceptor. Substrate mapping studies have demonstrated that glycosyltransferases are remarkably tolerant in the context of preparative synthesis. Mutagenesis can also broaden substrate recognition. Specific examples of the use of glycosyltransferases for the preparation of multivalent sialyl-Lewis X structures, toxin binding iso-globo trisaccharide, Pk-trisaccharide and blood group A and B trisaccharides and their analogs will be presented.

(3) **On the Regioselectivity in the Transglycosylation Using Glycosidases for the Enzymatic Syntheses of Glycans**

Katsumi Ajisaka.
265-1 Higashijima, Niitsu, Niigata 956-8603, Japan.

For the syntheses of glycans with complex structure, a chemical synthesis procedure has generally been the preferred method, but alternative enzymatic approaches have also been utilized in some cases. Both methods have distinct advantages but each also has practical disadvantages, and therefore a series of chemoenzymatic protocols, which combine aspects of both chemical and enzymatic synthesis procedures, are being developed to try to overcome previous technical limitations.

We have now developed a systematic chemoenzymatic method for the synthesis of glycopeptides.[1)] In this approach, either enzymatically or partly chemically synthesized oligosaccharide blocks are combined in a reaction that utilizes a chemical synthetic method to construct *N*-glycans. For *O*-glycan synthesis, a stepwise method has been adopted, whereby GalNAc-linked peptides are first generated with a peptide synthesizer, followed by the attachment of a galactose molecule to the GalNAc residue. This addition of galactose is catalyzed by recombinant β1,3-galactosidase from *Bacillus circulans*, which generates a β1,3-linkage via transglycosylation. As a final step in this procedure, sialic acid is attached via sialyltransferase to produce sialyl T-antigen linked glycopeptides.

For the application of the enzymatic component of this technique, it is essential that the selected glycosidases have a high level of regioselectivity, as it has been reported previously that transglycosylation reactions with glycosidases are generally not regioselective. However, we have shown that oligosaccharides can be synthesized regioselectively, if the appropriate enzyme is chosen. Examples of this includes, α-fucosidase from *Penicillium multicolor* which generates a fucosyl α1,3-linkage, and β1,3-galactosidase from *B. circulans* and β-galactosidase from *Bifidobacterium bifidum* which catalyze a galactosyl β1,3-linkage and a galactosyl β1,4-linkage, respectively, each with a high degree of regioselectivity. Hence, by use of a correctly selected purified glycosidase, the necessary linkage can be obtained in a high yield and with high specificity.

Recently, the transglycosylation specificity of β1,3-galactosidase from *B. circulans* was suspected to be non-regioselective when using galactose as the acceptor molecule. In our present study, we have thus re-examined the regioselectivity of various bacterial glycosidases with different acceptor molecules, as this had only been previously confirmed using either glucose or GlcNAc acceptors. We then performed transglycosylation reactions with variety of glycosidases using both galactose or mannose as acceptors[2)] and subsequently applied these enzymes to the synthesis of biologically important glycans.

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(4) **Combinatorial Chemical Approaches to Indentifying Non-natural Lectin Ligands**

Jacquelyn Gervay-Hague.
Department of Chemistry, One Shields Ave., Davis, CA 95616.

To overcome the challenges of complex oligosaccharide library synthesis, glycopeptides have been explored as mimics of oligosaccharides. These glycopeptides contain sugar moieties, which provide specificity by directing the ligand to the oligosaccharide binding site, and the peptide functions as a scaffold for optimal orientation of the glycan. Assembly of glycopeptides is considerably more facile than that of oligosaccharides and the process can be adapted to combinatorial synthesis with either glycosylated amino acid building blocks or by direct glycosylation of peptide templates. Combinatorial synthesis has developed into a useful method for the rapid identification of lead compounds for drug discovery. Identification of positive hits is often the most challenging aspect of this approach. With peptide libraries containing only natural amino acids, the sequence of active compounds has conventionally been achieved by Edman degradation sequence analysis. In the case of non-peptide libraries, encoding and decoding technologies are required. Ladder synthesis is one method of tagging, but in recognition processes it introduces the possibility that ladder fragments may interfere with receptor binding. To address this problem, we have developed a simple methodology for topologically segregating the tag on the interior of a bead, which is surface functionalized with a potential ligand. Thus, only the testing compounds are exposed to the target receptor. Here, we apply the methodology in the construction of glycopeptide combinatorial libraries for identification of high-affinity lectin ligands.

(5) **Chondroitin Polymerization is Achieved by Multiple Enzyme Complexes Consisting of Chondroitin Synthase Family Members**

Toru Uyama, Hiroshi Kitagawa, Tomomi Izumikawa and Kazuyuki Sugahara
Department of Biochemistry, Kobe Pharmaceutical University.

Chondroitin sulfate (CS) is a linear, sulfated polysaccharide composed of repeating disaccharide units of GalNAc and GlcUA residues, and synthesized onto the linkage tetrasaccharide attached to specific serine

residues in the core proteins. Recent experiments have revealed that various glycosyltransferases are responsible for CS biosynthesis[1-5]. To elucidate whether other unidentified glycosyltransferases are involved in CS biosynthesis, we screened the database using the amino acid sequence of chondroitin synthase-1 (ChSy-1)[1], which possesses GalNAcT-II and GlcAT-II activities responsible for elongation of CS but cannot polymerize chondroitin chains by itself, and discovered a novel protein. This protein shared several features with ChSy-1, type II transmembrane protein topology, two glycosyltransferase domains and similar genomic organizations, and was named chondroitin synthase-2 (ChSy-2). While we were analyzing functions of ChSy-2, Narimatsu and colleagues cloned the same gene and termed it chondroitin sulfate synthase-3[6]. However, the glycosyltransferase activities of ChSy-2 were quite low compared to those of ChSy-1[6]. On the other hand, we demonstrated that coexpression of ChSy-1 with chondroitin polymerizing factor (ChPF) showed dramatic augmentation of glycosyltransferase activities of ChSy-1 and also polymerization activities of chondroitin chains[5]. These results prompted us to evaluate effects of coexpression of ChSy-2 with ChPF, because ChSy-2 and ChSy-1 have similar properties. When ChSy-2 was coexpressed with ChPF in COS-1 cells, substantial GalNAcT-II and GlcAT-II activities were detected compared to ChSy-2 alone, which showed negligible glycosyltransferase activities. Furthermore, polymerization of chondroitin chains was demonstrated by coexpression of ChSy-2 with ChPF. Interestingly, coexpression of ChSy-2 with ChSy-1 also exhibited polymerization activity. In addition, lengths of chondroitin chains formed by coexpression in various combinations of these proteins were different. In pull-down assays, any two members of ChSy-2, ChPF and ChSy-1 interacted with each other. Thus, chondroitin polymerization could be achieved by coexpression in multiple combinations of ChSy-2, ChSy-1 and ChPF, suggesting physiological significance of these molecular interactions.
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(6) Degree of Polymerization (DP) of Polysialic Acid (PolySia) on Neural Cell Adhesion Molecules: Development and Application of a New Strategy to Accurately Determine the DP of PolySia Chains on N-CAM

Daisuke Nakata and Frederic A. Troy II
Department of Biochemistry and Molecular Medicine, University of California School of Medicine, Tupper Hall, Davis, California 95616.

The α2,8-linked polysialic acid (polySia) chains are structurally unique anti-adhesive glycotopes that in vertebrates, covalently modify the N-glycans of neural cell adhesion molecules (N-CAMs). These chains play a key role in modulating cell-cell interactions, principally during embryonic development. Two distinct polysialyltransferases (polySTs) are involved in the biosynthesis of polySia and both have the capability to polysialylated N-CAM. Several studies have attempted to determine the degree of polymerization (DP) of the polySia chains on N-CAM, a posttranslational modification that is postulated to be of critical importance in understanding N-CAM function. There are major limitations, however, in the conventional methods to accurately determine the DP of the parent polySia chains on N-CAM. The most serious is the partial acid hydrolysis of the labile α2,8-ketosidic linkages that occur during labeling procedures to tag the chains, which is necessary to enhance chromatographic detection. To determine the DP of the parent polySia chains on N-CAM, we have developed a facile method that combines the use of endo-β-galactosidase (EβGalase) to first release individual polySia chains from N-CAM, with high resolution HPLC profiling. The amount of Neu5Ac in each HPLC fraction is then quantitatively determined by DMB (1,2-**d**iamino-4,5-**m**ethylenedioxy**b**enzene) derivatization. This strategy avoids acid hydrolysis of polySia prior to chromatographic profiling and thus provides an accurate determination of the DP of the parent chain. To evaluate the potential power of this new method, a non-polysialylated construct of N-CAM was polysialylated in a reconstituted *in vitro* system by a soluble construct of STX or PST, and analyzed. While most of the oligo- polySia chains consisted of DPs ~50-60 or less, a population of chains with DPs ~150 and ~180 were detected. Surprisingly, a smaller population of chains with DPs >200 were observed. The diagnostic enzyme for identifying polySia, Endo-N-acylneuraminidase (Endo N), was used to confirm the nature of these extended chains. This experimental method has thus revealed a sub-population of polySia chains with DP values considerably greater than reported previously for N-CAM, and which are similar to the DP described earlier for the polySia capsule of neuroinvasive *E. coli* K1. An important and unexpected corollary of these studies is that the polySTs do not need to be intercalated into the membrane to catalyze synthesis of full length polySia chains on N-CAM. (Supported by NIH Research Grant GM 55703 to F.A.T.)

(7) Biological Function of Mucins Through Scavenger Receptor Present on Monocytes/Macrophages

Hiroshi Nakada, Munetoyo Toda, Hiroaki Takagi, Tatsuro Shigenobu, Masanobu Yoshida, Takaaki Inaba, Noriko Takeuchi and Mizue Inoue
Dept. Biotechnology, Kyoto Sangyo University

Mucins produced by cancer cells are found in the tissues and/or sera of cancer patients. There have been many reports that patients with a higher amount of mucins in their bloodstream have a lower 5 years survival rate. However, little is known regarding the biological significance of mucins. Mucins make contact with various circulating cells of the bloodstream in cancer patients or with the infiltrated cells in cancer tissues. We have demonstrated that mucins could activate monocytes/macrophages through a scavenger receptor (SCR), resulting in induction of cyclooxygenase 2 (COX 2) and subsequent overproduction of PGE2*. Up-regulation of COX 2 and overproduction of PGE2 have been implicated in the initiation and/or progression of colon cancer. It is important to make it clear in which cells and how COX 2 is induced initially in the tumor microenvironment. To further confirm the binding of mucins to SCR, we constructed FLAG tagged mouse SCR cDNA coding the ectodomain of SCR and prepared soluble SCR. The soluble SCR could bind to poly I but not to poly C, which is known to be a characteristic of SCR-ligand. It is also confirmed that soluble SCR bound to mucin-Sepharose. When mouse peritoneal macrophages were incubated with mucins in the presence of soluble SCR, production of PGE2 was inhibited, indicating that SCR is responsible for the binding to mucins. It is well-known that PGE2 plays various roles as a chemical mediator, leading to induction of VEGF, immunesupression and inhibition of apoptosis. Next, we examined the effects of mucins on tumor growth using two cell sublines named TA3-Ha and –St, which are derived from mouse mammary adenocarcinoma. It has been reported that TA3-Ha cells produce a mucin named epiglycanin but the latter does not. The two cell sublines showed a similar growth rate in vitro, but when each cell was injected into mouse subcutaneously, TA3-Ha tumor grew faster than TA3-St tumor did. In TA3-Ha tumor tissues, mucins were expressed and COX 2 was remarkably induced. Although similar number of macrophages were infiltrated in both tumor tissues, COX 2 in macrophages was induced in TA3-Ha tumor tissues but not in TA3-St tumor tissues. It is also noted that angiogenesis was prominently observed in TA3-Ha tumor tissues but not in TA3-St tumor tissues. It has been reporterd that PGE2 is one of mediators to induce VEGF and MMP-2. We also compared the levels of VEGF mRNA, MMP-2 mRNA and active form of MMP-2. All levels of these factors were elevated in TA3-Ha tumor tissues. Thus, we propose the following cascade in the tumor microenvironment. Mucins are produced by cancer cells. Infiltrated macrophages are activated by the mucins through the SCR. PGE2 secreted from the macrophages binds to EP2 receptor present on cancer cells and/or other cells, and activated cancer cells and macrophages produce VEGF as reported by M. Sonoshita et al. (Nat. Med. 7, 1048, 2001), leading to favorable conditions in epithelial cancer tissues for cancer cell growth. * T. Inaba et al. Proc. Natl. Acad. Sci. USA, 100: 2736-2741, 2003

(8) Structural and Carbohydate Binding Properties of Galectins

C. Fred Brewer.
Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461.

Galectins are a family of animal lectins with conserved carbohydrate recognition domains that possess nominal binding specificity toward LacNAc residues. The structures of galectins, however, differ in certain cases, as do their biological properties. For example, galectin-1, which is dimeric, possesses apoptotic activity, while galectin-3, which is primarily monomeric in solution, is often reported to possess antiapoptotic activity. In order to understand the structure-function basis of the different biological properties of galectins, we have undertaken a study of their physical and carbohydrate binding specificities including determining their quaternary structures in solution in the presence and absence of carbohydrates, their thermodynamics of binding to multivalent carbohydrates and glycoproteins, as well as their cross-linking properties with multivalent glycoconjugates. The concepts of homogeneous and heterogeneous cross-linking have been

recently introduced to help explain differences in the biological properties of galectin-1 and -3. More recent results will be presented that provide insight into the structure-function properties of galectins.

(9) **Galectin Binding to Glycoproteins and Glycans: Consequences of Low-Affinity, High-Avidity Interactions**

Linda G. Baum[1], Brianna Stillman[1], Joseph D. Hernandez[1], Alshakim Nelson[2], Jason M Belitsky[2], Daniel K Hsu[3], Fu-Tong Liu[3] and J. Fraser Stoddart[2]
[1] Dept. of Pathology and Laboratory Medicine, UCLA School of Medicine, [2] Dept. of Chemistry, UCLA, [3] Dept. of Dermatology, UC Davis School of Medicine.

Galectins can discriminate among a broad range of oligosaccharide ligands. However, the ability of galectins to recognize complex glycoprotein counterreceptors on the surface of cells does not always reflect the relative binding affinities of galectins for the isolated glycans that decorate these glycoproteins. To probe the biologic functions of different galectins that bind T cells, we have compared the effects of galectin-1 and galectin-3 binding on T survival and have identified the T cell surface glycoproteins that differentially bind galectin-1 and galectin-3. We have also examined the effects of modifying glycosylation of a major galectin-1 counterreceptor, CD43. In contrast to the relatively weak binding of galectin-1 to core 1 O-glycan sequences, we have found that galectin-1 can bind to CD43 bearing only core 1 O-glycans, as well as to CD43 bearing core 2 O-glycans. Finally, to understand the interaction of galectin-1 with high avidity ligands, we have examined the binding of galectin-1 to a novel multivalent polymer that allows rotational and translational flexibility in the presentation of lactose. These studies will illuminate the complex and subtle differences in galectin binding to different cell types, that can result in profoundly different biological outcomes.

(10) **Galectins and Innate Immune Responses: the Soluble β-Galactoside Binding Protein Family that can Modulate Immunity Through Their Proinflammatory Cytokine-Like Activities and as Pathogen Recognition Molecules**

Sachiko Sato.
Glycobiology laboratory, Research Centre for Infectious Diseases, CHUL Research Centre, Quebec, Canada.

The innate immune response is one of the premier defense systems that maintain individual integrity. This immunity is required to be initiated immediately after invasion of pathogens when they have the potential to compromise host integrity. In the absence of acquired immunity, innate immunity is able to sense the presence of "danger" either by detecting cell damage or by recognizing pathogens. Recent intriguing findings indeed suggest that innate immunity is capable of initiating a response to host intracellular components, which could be extracellularly released when cells are damaged by invading pathogens. Unlike other lectin families, galectins, a family of β-galactoside binding proteins, are uniquely synthesized as soluble cytosolic proteins, therefore they are not usually expressed extracellularly. Once tissues are injured by pathogens, galectins are expected to be released passively. In addition, it has been also well established that some galectins, such as galectin-3 and -9 are actively secreted by inflammatory macrophages or by activated lymphocytes. Those leukocytes are found in sites where infection has not been controlled by initial immune responses. Thus, either through passive release or by active secretion, some galectins appear outside of cells when innate immunity is required to be ignited. Importantly, series of studies on the extracellular roles of galectins raise a possibility that galectins could modulate immune/inflammatory responses. Indeed, others and we have found that some galectins trigger emigration of phagocytes, especially neutrophils, one of the first and dominant leukocytes recruited from peripheral blood to infected sites. Our recent results also suggest that galectin-1, -3 and -9 rapidly induce massive production of TNF-β, IL-1β and some chemokines that attract neutrophils and monocytes in vivo and in vitro. Therefore, galectins have a potential to act as proinflammatory cytokines that initiate innate immune responses. While interplay of various proinflammatory cytokine-like activities has an impact on immunity, recognition of invading pathogens by the system also play critical roles in the pathogenesis of infectious diseases. Importantly, unlike other type of cytokines, galectins also act as soluble adhesion molecules by cross-linking two different entities, resulting in mediation of cell-cell and cell-pathogen interactions. However, galectins are considered to be designed to recognize "self" glycans on host cells rather than "non-self" glycans on pathogens since galectins preferentially binds to lactosamine-containing oligosaccharides. Nevertheless, others and we have recently suggested that some parasite-unique oligosaccharide epitopes, such as $(Gal\beta1\text{-}3)_n$ residues of protozoa *Leishmania major* and GalNAcβ1-4GlcNAc residues of helminthes are recognized by galectin-3 and -9, and by galectin-3, respectively. In the case of *L. major*, galectin-9 promotes parasite adhesion as well as alters some cytokine synthesis by macrophages. Therefore, galectins are also able to be involved as pathogen-recognition molecules in immunity. In the case of viral pathogens, enveloped virus that abundantly contains "self" glycans on the surface could be recognized by galectins. Indeed, we recently found that low dose of galectin-1, but not galectin-3 promotes HIV-1 infectivity by facilitating viral adhesion to CD4 T lymphocytes. In conclusion, galectins act as unique cytosolic "lectinocytokines" or/and as pathogen recognition molecules to modulate innate immune responses to infections.

(11) **GlcNAc 6-*O*-Sulfotransferase (GlcNAc6ST) -1 and GlcNAc6ST-2 Regulate Lymphocyte Homing to Lymph Nodes**

Kenji Uchimura[1], Mark S. Singer[1], Durwin Tsay[1], Kenji Kadomatsu[2], Reiji Kannagi[3], Takashi Muramatsu[2] and Steven D. Rosen[1]
[1] Department of Anatomy, Program in Immunology, University of California, San Francisco, CA 94143, [2] Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan, [3] Program of Molecular Pathology, Aichi Cancer Center, Nagoya 464-8681, Japan.

Lymphocytes migrate between the blood and lymph nodes (LN). In the first step of lymphocyte homing to LN, tethering and rolling of lymphocytes are initiated by the binding of L-selectin on lymphocytes to its carbohydrate-modified ligands, such as CD34, on high endothelial venules (HEV). Sialyl 6-sulfo Lewis X is a major capping group of L-selectin ligands on HEV. GlcNAc-6-sulfation within this structure is a pertinent modification for the ligand activity, and is catalyzed by GlcNAc 6-*O*-sulfotransferases (GlcNAc6STs) of which GlcNAc6ST-1 and GlcNAc6ST-2 (HEC-GlcNAc6ST/LSST) are expressed in HEV. Our previous studies in vitro showed that GlcNAc6ST-1 and GlcNAc6ST-2 have the ability to reconstitute sialyl 6-sulfo Lewis X and the epitope of MECA-79, a function-blocking antibody that reacts with L-selectin ligands on HEV. Genetic deletion of GlcNAc6ST-2 resulted in the strong reduction of the MECA-79 epitope in LN-HEV and a 50% reduction of L-selectin-mediated lymphocyte homing to peripheral lymph nodes (PLN). These data established the importance of GlcNAc6ST-2 for lymphocyte homing in vivo. However, clearly there must be GlcNAc6ST-2 independent ligands in LN. We have recently produced and analyzed mice deficient in GlcNAc6ST-1 gene. GlcNAc6ST-1 null mice were also impaired in the elaboration of sialyl 6-sulfo Lewis X and the MECA-79 epitope in HEV. Lymphocyte homing to PLN was significantly reduced (22% reduction) in GlcNAc6ST-1 null mice. GlcNAc6ST-1 as well as GlcNAc6ST-2 has been shown as an enzyme involved in lymphocyte homing in vivo. Each null strain has been backcrossed into C57BL/6 mouse strain by confirming 104 microsatellite genetic markers. Breeding the backcrossed strains has generated GlcNAc6ST-1/GlcNAc6ST-2 doubly null mouse. We have found that the MECA-79 epitope in HEV of LN and Peyer's patches is totally abolished, and that lymphocyte homing to PLN is greatly diminished (~75% reduction) in the doubly null mice. These results indicate that GlcNAc6ST-1 and GlcNAc6ST-2 play complementary roles in lymphocyte homing in vivo. Possible mechanisms for the residual homing in the doubly null mice are being explored.

(12) **H-Ras Induces FucT-VII expression via Raf and PI3K**

Dimitrios G. Zisoulis and Geoffrey S. Kansas
Dept. of Microbiology-Immunology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Ave, Chicago, IL 60611.

Expression of the α(1,3)-fucosyltransferase FucT-VII is essential for the biosynthesis of ligands for the E-, P- and L-selectins. FucT-VII expression in myeloid cells is constitutive, but in T cells it is inducible and highly regulated. Yet little is known about the mechanisms which govern FucT-VII induction in T cells or other lymphocytes. Studies from our lab have revealed that enforced expression of constitutively active Ras in Jurkat T cells leads to transcriptional upregulation of FucT-VII, in part by triggering the Raf-MEK-ERK signaling cascade. Furthermore, our data reveal Ras isoform specificity in FucT-VII induction, since retroviral transduction of cells with active H-Ras, but not with N- or K-Ras, leads to FucT-VII upregulation. Pharmacologic inhibition of the phosphoinositide-3 kinase (PI3K) activity abolished H-Ras-mediated FucT-VII induction, implicating PI3K as a FucT-VII regulator. However, Ras activation of PI3K is

superfluous in this system, since Jurkat cells are deficient in PTEN, which opposes the activity of PI3K, thus resulting in strong constitutive PI3K activity. Equivalent levels of PI3K activation and ERK phosphorylation are seen in cells expressing each of the three Ras isoforms, indicating that neither differential PI3K activity nor differential ERK activation can explain the selective ability of H-Ras to trigger FucT-VII expression. Interestingly, only H-Ras strongly upregulates the activity of the AP-1 transcription factor, suggesting that activation of AP-1 may be responsible for the observed Ras isoform specificity of FucT-VII induction. To gain further insight into Ras effectors involved in FucT-VII regulation, we analyzed the ability of H-Ras effector-loop domain mutants, which are selectively impaired in their ability to interact with certain downstream effectors while retaining interaction with other effectors, alone or in combination with other activated signaling proteins, to induce FucT-VII expression. Activation of the PI3K, Raf or RalGDS signaling pathways alone, in the presence of strong constitutive PI3K activity, failed to upregulate FucT-VII, in agreement with the involvement of at least two signaling pathways. In addition, the combination of active Raf with any of the H-Ras effector loop mutants was also unable to induce FucT-VII expression. These studies show that H-Ras mediates FucT-VII induction via the concomitant activation of the Raf, PI3K and a third, H-Ras-specific, signal transduction pathways.

(13) **Strategies for Phosphomannosyl Recognition**

Nancy M. Dahms and Jung-Ja P. Kim
Medical College of Wisconsin, Department of Biochemistry, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226.

The 46kDa cation-dependent mannose 6-phosphate receptor (CD-MPR) and the 300kDa cation-independent mannose 6-phosphate/insulin-like growth factor II (IGF-II) receptor (CI-MPR) are the sole members of the P-type lectin family and are distinguished from all other lectins by their ability to recognize phosphorylated mannose residues. These receptors play an essential role in the generation of functional lysosomes within the cells of higher eukaryotes by directing newly synthesized lysosomal enzymes bearing mannose 6-phosphate (Man-6-P) from the trans Golgi network to lysosomes. In addition to its role in lysosome biogenesis, the CI-MPR has been implicated in several other cellular processes, including cell growth, apoptosis, and cell migration, due to its ability to bind a wide range of Man-6-P-containing (e.g., latent transforming growth factor-beta, granzyme B, proliferin) and non-Man-6-P-containing (IGF-II, retinoic acid, urokinase-type plasminogen activator receptor, plasminogen) molecules at the cell surface. The ability of the CI-MPR to interact with many different proteins and a lipophilic molecule is facilitated by the receptor's ~2,270-residue extracytoplasmic region comprised of 15 homologous domains in which several distinct ligand binding sites have been localized to individual domains. Our studies have provided a detailed view of the mechanism of high affinity (nM) phosphomonoester recognition by both MPRs that reveal different binding-site architectures. Furthermore, our recent biochemical studies demonstrate that the CI-MPR contains a third Man-6-P recognition site that is located in domain 5 and exhibits lower affinity than the carbohydrate-binding sites present in domains 1-3 and 9. The identification of a third Man-6-P binding site along with the crystal structure of the N-terminal region of the CI-MPR has allowed us to generate a model of the entire extracytoplasmic region and its interaction with a multivalent ligand: the proposed flexible nature of the CI-MPR, involving extended, bent, and dimeric conformations, would facilitate the presentation of its carbohydrate-binding sites (i.e., domains 3, 5, and 9) in various spatial arrays that could serve to match the diverse display of Man-6-P-containing oligosaccharides found on its numerous ligands.

(14) **Endo-β-Mannosidase, A Plant Enzyme Acting on *N*-Glycan: Purification, Substrate Specificity, and Molecular Cloning**

Takeshi Ishimizu[1], Akiko Sasaki[1], Satoshi Okutani[1], Mami Maeda[1], Mai Yamagishi[1,2] and Sumihiro Hase[1]
[1] Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan,
[2] (present address) Major in Integrative Bioscience and Biomedical Enginnering, Waseda University, Okubo, Tokyo 169-8555.

Endo-β-mannosidase is a novel endoglycosidase which hydrolyzes the Manβ1-4GlcNAc linkage in the trimannosyl core structure of *N*-glycans (1). Here we report the purification, substrate specificity, and molecular cloning of endo-β-mannosidase (2). The enzyme purified from lily flowers gave a single band on native-PAGE and three bands on SDS-PAGE with molecular masses of 42, 31, and 28 kDa. The purified enzyme hydrolyzed pyridylaminated (PA-) $Man_nMan\alpha1$-6Manβ1-4GlcNAcβ1-4GlcNAc (n = 0~2) to $Man_nMan\alpha1$-6Man and GlcNAcβ1-4GlcNAc-PA. It did not hydrolyze PA-sugar chains containing Manα1-3Manβ or Xylβ1-2Manβ. The best substrate in the PA-sugar chains investigated was Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA with K_m value of 1.4 mM. The enzyme, however, more preferred a corresponding glycopeptide, Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-peptide (K_m value 75 mM) as a substrate, indicating that this enzyme recognized not only the vicinity of the Manβ1-4GlcNAc linkage but also around non-reducing end of the sugar chain including the peptide portion. Based on the amino acid sequence information of the purified enzyme, cDNAs encoded endo-β-mannosidase from lily and arabidopsis were cloned, and the encoded proteins were consisted of 952 and 944 amino acid residues, respectively. Three polypeptides of the lily endo-β-mannosidase were encoded by this single gene and were generated by post-translational proteolysis. The arabidopsis enzyme expressed in *Escherichia coli* had similar substrate specificity of that of the lily enzyme. Endo-β-mannosidase belonged to the clan GH-A of glycosyl hydrolases. Site-directed mutagenesis experiments revealed that two glutamic acid residues (Glu464 and Glu549 of the arabidopsis enzyme) conserved in this clan were critical for enzyme activity. Amino acid sequences of endo-β-mannosidases showed distinct difference from those of the bacterial, fungal, and animal exo-type β-mannosidases. Indeed, endo-β-mannosidase-like genes were only found in plants, indicating that endo-β-mannosidase is a plant specific enzyme. The enzyme was ubiquitously expressed in all the lily organs investigated. The endo-β-mannosidase was not an organ specific enzyme and might have a general role for plant cells such as processing and/or degradation of *N*-glycans. (1) Sasaki, A. et al. (1999) *J. Biochem.* 125, 363-367; (3) Ishimizu, T. et al. (2004) *J. Biol. Chem.* 10.1074/jbc.M406886200

(15) **NMR Structural Biology of the Intracellular Lectins that Contribute to Quality Control of Glycoproteins in Cells**

Koichi Kato.[1,2]
[1] Nagoya City University, [2] CREST/JST.

Accumulating evidence shows that carbohydrate moieties covalently linked to proteins contribute to polypeptide folding, transport, and degradation in cells via interactions with a variety of intracellular lectins. To provide structural basis of molecular recognition of glycoproteins in those systems, we performed stable-isotope-assisted NMR analyses of structures, dynamics, and sugar-binding of the intracellular lectins, which include the ER chaperones calreticulin and calnexin, the cargo receptors VIP36 and ERGIC-53, and the ubiquitin ligase SCF^{Fbs1}. The sugar residues involved in the lectin-binding were identified on the basis of NMR spectral data of high-mannose type oligosaccharides titrated with the carbohydrate recognition domains (CRDs) of those lectins produced by *Escherichia coli* expression systems. The amino acid residues located in the ligand-binding sites of the CRDs were identified based on chemical shift perturbation data. Inspection of nuclear Overhauser effect (NOE) data allowed us to determine conformations of oligosaccharides or glycopeptides in association with the CRDs and to characterize ligand-lectin contacts at atomic resolution. In those analyses, stable isotope (^{13}C and ^{15}N) labeling of the CRDs as well as their cognate ligands plays key roles. For example, using the stable-isotope-assisted NMR method, we revealed that the CRD of SCF^{Fbs1} binds the carbohydrate-polypeptide junction of its substrates, which is hidden in a native glycoprotein but is likely to be exposed in unfolded glycoproteins doomed to ubiquitin/proteasome-mediated degradation.

Reference

Mizushima, T. *et al.*, (2004) Structural basis of sugar-recognizing ubiquitin ligase. *Nature Struct. Mol. Biol.* **11,** 365-370.

(16) **Mutations in the GlcNAc-Phosphotransferase α-/β-Subunits Precursor Gene are the Molecular Basis of Both Mucolipidosis II and IIIA**

Mariko Kudo, Courtney P Kerbo and William M Canfield
Genzyme, 800 Research Parkway, suite 200, Oklahoma City, OK 73104.

The trafficking of most lysosomal hydrolases in higher eukaryotes is mediated by a mannose 6-phosphate (M6P) dependent pathway. GlcNAc-phosphotransferase is the enzyme that catalyzes the initial step of the biosynthesis of M6P by transferring GlcNAc-phosphate to the high-mannose type glycans on newly synthesized lysosomal hydrolases. Mutations in GlcNAc-phosphotransferase are believed to be the cause of the lysosomal trafficking diseases mucolipidosis II (ML II, I cell disease),

classical mucolipidosis III (ML IIIA, pseudo-Hurler polydystrophy), and variant polymucolipidosis III (ML IIIC). GlcNAc-phosphotransferase activity is absent in ML II and decreased in ML IIIA. In ML IIIC, GlcNAc-phosphotransferase activity is altered with respect to lysosomal hydrolases but not synthetic substrates. GlcNAc-phosphotransferase is a complex enzyme with a subunit structure $\alpha_2\beta_2\gamma_2$. Previously, we demonstrated the enzyme is the product of two genes, one encoding the γ -subunit and the second the α- and β-subunits. Further studies revealed that mutations in the γ-subunit caused ML IIIC. This finding strongly suggested that the catalytic domain of the enzyme is located on the α-and/or β-subunits, while the γ-subnit plays a role in recognition of protein substrates. To understand the genetic and molecular basis of ML II and ML IIIA, we sequenced the cDNA and genomic DNA for the α-/β-subunits precursor gene, and analyzed mutations in ML II and ML IIIA patients and their families. We obtained fibroblasts or lymphocytes of ML II or ML IIIA patients and their families from cell depositories and collaborators. First, GlcNAc-phosphotransferase activity of these cells was determined to confirm the phenotype of the cells. Then each of the 21 exons and surrounding introns of the α-/β-subunits precursor gene was sequenced to identify mutations. Additional characterization of mRNA by RT-PCR was performed to confirm potential splicing defects. cDNAs corresponding to some of the mutants were expressed in mammalian cells to confirm that the mutation altered the enzyme activity. These studies have identified fifteen different mutations including substitutions deletions, and insertions. Two mutations were identified in each patient. When parent samples were available, one of the mutations was also present in each parent DNA. Mutations in the α-/β-subunits precursor gene were identified in each patient with ML II or ML IIIA. Mutations causing early termination of the α-/β-subunit precursor translation products were identified in MLII patients, consistent with the absence of GlcNAc-phosphotransferase activity observed in the fibroblasts. These included mutations caused by substitutions, and frame shifts caused by deletion, insertion or abnormal splicing. In contrast, in ML IIIA, mutations such as amino acid substitutions and incomplete splicing were found, consistent with the decreased but detectable GlcNAc-phosphotransferase activity observed in the fibroblasts. Some ML IIIA patients were compound heterozygotes which had both a severe (ML II-like) mutation and a milder (ML IIIA-like) mutation. As expected, no mutations were found in the single ML IIIC patient studied. These studies identify the molecular basis of ML II and ML IIIA, and clarify the basis for distinguishing these related diseases of lysosomal enzyme trafficking.

(17) **Unique *O*-glycan Biosynthesis in *Trypanosoma cruzi* Glycoproteins**

Lucia MendonÁa-Previato, Adriane R. Todeschini, Wagner B. Dias, Orlando A. Agrellos, Norton Heise and JosÈ O. Previato

Instituto de Biof'sica Carlos Chagas Filho, Centro de Ci'ncias da Sa˙de, Universidade Federal do Rio de Janeiro, Cidade Universit·ria, 21944-970, Rio de Janeiro-RJ, Brasil.

Trypanosoma cruzi, the parasitic protozoan responsible for Chagas' disease in Latin America, consists of a heterogeneous group of strains that infect a wide range of mammalian hosts, present distinct tissue-tropism and cause several clinical manifestations in the course of infection, leading to death or serious damage to the heart, digestive tract or nervous system during the chronic phase of the disease. The causes of this wide variability are not known. Recently, based on multilocus enzyme electrophoresis, random amplified polymorphic DNA and on structural and functional variation of 24Sα rDNA and mini-exon genes, *T. cruzi* isolates have been subdivided into two major phylogenetic lineages named *T. cruzi* 1 and *T. cruzi* 2. Although each group still retains considerable heterogeneity, a correlation between the different clinical manifestations and the genetic background of the parasite was also roughly achieved. The variability observed during infection by different *T. cruzi* strains may also be presumably related to the overall structure of macromolecules expressed on both the parasite and host cell surface, since *T. cruzi* is an intracellular parasite and must invade cells of the mammalian host in order to replicate and liberate infective forms to complete its life cycle. Indeed, *T. cruzi* surface sialoglycoproteins, known as mucin-like molecules, are implicated in the interaction of the parasite with host cells and the modulation of the host immune system. The extensive *O*-glycosylation of these molecules leads to the production of variable molecular mass mucins found in all stages of the parasite. Structural characterization of *O*-glycans from *T. cruzi* epimastigote mucins has been reported and revealed unique features when compared to mammalian mucins: (i) *T. cruzi O*-linked glycans are always attached to the peptide by α-GlcNAc-*O*-Thr linkages; (ii) depending on the group, the *O*-4 position of the α-GlcNAc residue can be initially substituted by either β-Gal*f* (*T. cruzi* 1) or β-Gal*p* (*T. cruzi* 2); (iii) the *O*-4 substituted α-GlcNAc is then elongated by a β-Gal*p*1→6 branch; and (iv) one of the terminal Gal residues can be finally modified by addition of α-Neu5Ac2→3 catalyzed by a *trans*-glycosylation reaction. The presence of these structures implies the participation of a novel set of glycosyltransferases in *T. cruzi O*-glycan biosynthesis and provide insights for targets that can be used for the development of new and potential trypanocidal drugs. Supported by CNPq, and FAPERJ

(18) **hGPI7 Regulates the Generation of GPI-anchors by Competing for PIG-F with PIG-O**

Nobue Shishioh[1], Yeongjin Hong[2], Yusuke Maeda[1] and Taroh Kinoshita[1]

[1] Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan., [2] Genomic National Center for Enteropathogenic Bacteria and Department of Microbiology, Chonnam National University Medical School, Gwangiu, S.Korea..

Many eukaryotic cell surface proteins are anchored to the membrane via glycosylphosphatidylinositol (GPI). GPI precursor is synthesized by sequential additions of glucosamine, mannoses (Man), and ethanolamine phosphates (EtNP) onto phosphatidylinositol. The complete precursor to be attached onto nascent proteins is termed H7 in mammalian cells, and is synthesized by PIG-O, a transferase of EtNP to the third Man of an intermediate termed H6. H7 can be further modified to become H8 by attachment of an additional EtNP to the second Man. However, it is unclear whether modification of the second Man with EtNP is important for the surface expression of GPI-anchored proteins (GPI-APs) in mammalian cells. There is a report that yeast *GPI7* is involved in the transfer of EtNP to the second Man, whereas the gene responsible in mammalian cells has not been identified. In this study, we cloned human homologue of GPI7 (hGPI7). To examine its role in mammalian GPI biosynthesis, we established a cell line with a reduced level of hGPI7 mRNA by RNA interference. We examined the GPI biosynthesis of the knock-down cells and found that these cells accumulated H7 with little production of H8. These results suggested that hGPI7 was involved in transfer of EtNP to the second Man. Despite the reduced availability of H8, the expression level of GPI-APs on the surface of the knock-down cells was unaffected, indicating that the addition of EtNP to the second Man is not necessary for the surface expression of GPI-APs in mammalian cells. Interestingly, catalytic activity of PIG-O was apparently enhanced as evidenced by the decrease of H6 concomitant with the increase of H7 in hGPI7 knock-down cells. We considered the possibility that hGPI7 is somehow involved in regulating PIG-O catalytic activity. To begin with, we examined if hGPI7 binds to PIG-O or PIG-F, a component of PIG-O complex. Immunoprecipitaion experiments demonstrated that hGPI7 bound to PIG-F but not PIG-O. To test if hGPI7 is stabilized by PIG-F, we expressed hGPI7 in PIG-F mutated cells. The expression level of hGPI7 in PIG-F mutated cells was much lower than in rescued cells. These results suggested that hGPI7 is bound to and stabilized by PIG-F. Because PIG-O forms a complex with PIG-F and the complex formation is important for the stability of PIG-O, we hypothesized that hGPI7 competes with PIG-O for PIG-F binding. If so, the overexpressing hGPI7 would destabilize PIG-O, resulting in less production of H7. As expected, overexpression of hGPI7 resulted in a decreased expression level of PIG-O. Furthermore the overexpressing cells accumulated H6 and produced a lower level of H7. We propose a novel regulation of GPI biosynthesis by the competition between two independent enzymes, PIG-O and hGPI7, for one regulatory protein PIG-F.

(19) **Cytosolic Free Oligosaccharides: Formation and Cytosolic Processing.**

Tadashi Suzuki.

2-2 Yamada-oka, Suita-shi, Osaka 565-0871, Department of Biochemistry, Osaka University Graduate School of Medicine/PRESTO, JST.

It has been shown that cytoplasmic peptide:*N*-glycanase (PNGase)-catalyzed deglycosylation occur during the ERAD (endoplasmic reticulum-associated degradation) process of misfolded/unassembled glycoproteins. The deglycosylation process by PNGase is believed to be critical for subsequent proteolysis by the 26S proteasome. Using yeast system (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) we are studying the functional importance of this enzyme and phenotypic consequence of defect of this enzyme. In this symposium the current knowledge on functional importance of PNGase will be summarized.

Once free oligosaccharides are released by PNGase in the cytosol, they have to be catabolized in the cytosol before they are finally transported into the

lysosome for further degradation. Mammalian cells has a very sophisticated, organized pathway for their processing of free oligosaccharides, though the molecular nature of it remains largely unknown. Recently genes encoding enzymes involved in this process have been identified. Potential importance of glycan processing in the cytosol will also be discussed.

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(20) Protein *O*-Fucosyltransferase 1(*O*-FucT-1) is a Soluble Enzyme Localized to the ER

Yi Luo, Kelvin B. Luther and Robert S. Haltiwanger
Department of Biochemistry and Cell Biology, Institute of Cell and Developmental Biology, SUNY-Stony Brook, Stony Brook, NY 11794-5215.

LAD II/CDG IIc is a rare autosomal recessive disease characterized by leukocyte adhesion deficiency as well as severe neurological and developmental abnormalities. The molecular defect in LADII/CDG IIc is in the Golgi GDP-fucose transporter, resulting in decreased GDP-fucose levels in the Golgi lumen and, hence, reduction of fucosylated antigens on the cell surface. A recent study using fibroblasts from LADII/CDG IIc patients suggested that while terminal fucosylation of N-glycans is reduced severely, protein *O*-fucosylation is generally unaffected (Sturla *et al*. 2003 *J. Biol. Chem.* **277**:26727). A potential explanation for this phenomenon is that enzymes adding *O*-fucose localize to cell organelles other than the Golgi apparatus. In this study, we investigated the subcellular localization of Protein *O*-fucosyltransferase 1(*O*-FucT-1), responsible for adding *O*-fucose to EGF repeats. Re-examination of the amino terminal residues of mouse, human and *Drosophila O*-FucT-1 suggested that they may encode a signal sequence rather than a type II transmembrane sequence. Biochemical analysis reveals that more than 90% of *O*-FucT-1 activity is found in high speed supernatants, consistent with this observation. Assays of sucrose gradient fractions of microsomes from rat liver revealed that the *O*-FucT-1 activity co-localizes with the ER marker Glucose 6-phosphatase but not the Golgi marker β4GalT. Immunofluorescent staining of COS1 cells transiently transfected with a plasmid expressing an N-terminal tagged human *O*-FucT-1 revealed that the expressed protein co-localizes with an ER marker(calreticulin) but not with a Golgi marker(GM130). Interestingly, deletion of a KDEL-like 'RDEF' sequence at the C-terminus of human *O*-FucT-1 results in loss of ER localization and secretion of *O*-FucT-1 into the media. These results strongly indicate that the RDEF sequence is functioning as a KDEL-like ER retention signal. We next attempted to elucidate whether the *O*-fucosylation reaction also occurs in the ER. We over-expressed mouse Notch1 EGF29-36 fragments in COS1 cells and metabolically labeled the cells with [^{3}H]-fucose. Immunofluorescence and sensitivity to endoglycosidase H digestion confirmed that EGF29-36 within the cells is localized to the ER. EGF29-36 secreted to the media acquired endoglycosidase H resistance. By comparing the stoichiometry of *O*-fucosylation using western blots and fluorography, we found that radioactive *O*-fucose levels on EGF29-36 from lysate and media were similar. Thus, unlike most known fucosyltransferases, *O*-FucT-1 appears to transfer fucose to substrates in the ER instead of Golgi . Taken together, our results suggest a previously unexpected characteristic of *O*-FucT-1 as a soluble, ER localized enzyme. Together with the fact that *O*-FucT-1 only recognizes properly folded EGF repeats, these results suggest that *O*-FucT-1 may play a role in quality control. This work was supported by NIH grant GM61126.

(21) Fractionation of the Mammalian Golgi Apparatus

Tomohiko Taguchi.
Yamadaoka 2-2, Suita, Osaka, Japan.

The Golgi apparatus is a highly polarized organelle in animal cells. Newly-synthesized proteins from the endoplasmic reticulum enter at the cis face of the Golgi apparatus, the cis-Golgi network, then they move through the stack to the trans-Golgi network where they are sorted to their different destinations both within and outside the cell. Passage through the Golgi stack is often accompanied by post-translational processing, most often to the bound oligosaccharides. Processing of *N*-linked oligosaccharides is the best characterized and involves the trimming of the high mannose oligosaccharides by mannosidases, followed by the addition of sugars that include *N*-acetylglucosamine, galactose, sialic acid and fucose, to generate complex oligosaccharides. These modifying enzymes appear to be arrayed across the Golgi stack in the order in which they work, such that transiting cargo is exposed to sequential modifications. The mechanism of cargo movement through the Golgi stack is still under debate. The two most popular models are the cisternal maturation model and vesicle-mediated transport. In both cases, however, COPI transport vesicles move from cisterna to cisterna in the stack, in the anterograde and/or retrograde direction. Targeting involves SNARE proteins and an array of tethers that comprise rab GTPases, fibrous, coiled-coil proteins, and multi-protein complexes. Most of these proteins are also distributed in a polarized manner across the stack helping to provide the required directionality for vesicle transport.

The distribution of the targeting proteins and the modifying enzymes across the Golgi has largely been determined using microscopic techniques, mostly EM techniques, both cytochemical and immunological. Immunological methods have relied on antibodies to endogenous proteins, or, when this has failed, antibodies to tags on expressed proteins. The result has been a two-dimensional map of proteins in the animal cell Golgi. There are, however, limitations to this map that reflect the sensitivity of the methods used. Cytochemical methods are the most sensitive but they are not quantitative. Immuno-gold methods are quantitative but they are much less sensitive, especially when the copy number of the target protein is low. What is needed is an independent means of validating the EM data.

We have exploited the breakdown of the Golgi apparatus that occurs during mitosis to isolate sub-fractions using immunoaffinity methods. Rat liver Golgi stacks were treated with mitotic cytosol from HeLa cells and the fragments then incubated with antibodies immobilized on magnetic beads. Antibodies against the cis-Golgi marker, GM130, bound membranes that were depleted in the trans-Golgi network marker, TGN38, whereas antibodies against the cytoplasmic tail of TGN38 did the reverse. A range of other Golgi enzymes, SNAREs and tethers were also tested and were found to bind to anti-GM130 antibodies to an extent that reflected their proximity to cis-cisternae as determined by other techniques. This method should provide a useful complement to the immuno-EM methods presently used to map the Golgi apparatus. Biochemical experiments now enabled to perfom with purified Golgi membranes will be also discussed.

(22) Analysis and Manipulation of the Protein *N*-Glycosylation Pathway in the Baculovirus-Insect Cell System

Donald L. Jarvis, Jared J. Aumiller, Jason R. Hollister, Ziad S. Kawar, John Pilon and Nadia Vadaie
Department of Molecular Biology, University of Wyoming, Laramie, WY, USA.

Insect protein glycosylation pathways appear to be intermediate in complexity relative to lower eucaryotes, such as yeasts, and higher eucaryotes, such as mammals. Insects generally appear to perform the same early steps in protein *N*-glycosylation, including *N*-glycan assembly, transfer, and trimming, as lower and higher eucaryotes. However, they generally appear to lack some of the functions required for *N*-glycan elongation. Thus, the major processed *N*-glycans produced by insects are usually relatively simple paucimannose structures (Man3GlcNAc2-R) with or without core fucose residues. An important implication of these observations is that recombinant *N*-glycoproteins produced with the baculovirus-insect cell system might not have structurally authentic glycans. In fact, recombinant *N*-glycoproteins produced using the baculovirus-insect cell system typically lack the complex, terminally sialylated glycans of many native mammalian glycoproteins. These insect-derived products typically have paucimannose *N*-glycans at the sites occupied by complex, terminally sialylated *N*-glycans in the native mammalian products. In recent studies, we have been addressing this problem by using metabolic engineering methods to extend the protein *N*-glycosylation pathway of lepidopteran insect cell lines. These efforts have yielded transgenic insect cell lines that encode and express mammalian glycosyltransferases and enzymes involved in CMP-sialic acid biosynthesis. Relative to the parental lines, these new cell lines can still serve as hosts for baculovirus expression vectors and produce similar levels of recombinant glycoproteins. Unlike the parental insect cell lines, however, the transgenic lines can produce recombinant glycoproteins with complex, terminally sialylated *N*-glycans. The majority of this talk will focus on the creation and characterization of these transgenic insect cell lines. Our insect glycobiology research is also

dedicated to providing a better basic understanding of insect protein *N*-glycosylation pathways. Towards this end, we use molecular genetics to identify genes encoding *N*-glycan processing enzymes in lepidopteran insect cell lines and to characterize the biochemical functions of the products. We believe this approach is most likely to yield an unequivocal view of insect *N*-glycan processing capabilities. The last part of this talk will highlight some of our efforts in this area. One effort resulted in the molecular cloning and characterization of an insect fl4-*N*-acetylgalactosaminyltransferase, which functions in *N*-glycoprotein biosynthesis and possibly also functions in glycolipid biosynthesis. Another effort, still in progress, is designed to molecularly clone and characterize a processing fl-*N*-acetylglucosaminidase, which distinguishes the insect and mammalian protein *N*-glycosylation pathways.

(23) **Plant Sialobiology**

Lokesh Joshi, Miti Shah, Sasha Daskalova, Vinay J. Nagaraj, Chitra Prasanna, Jared Gerlach, Amy-Grace Smith, Marta Waddell, Charles R. Flynn and Linda C. Lopez

The Biodesign Institute at Arizona State University, Tempe AZ 85287.

Sialic acids are a diverse family of amino sugars. In higher animals and certain microorganisms, sialic acids play important roles in attenuating molecular interactions and determining protein half-life[1]. Until recently, it was accepted that plants were unable to carryout this particular post-translational modification. However, recent studies by our group have shown that plants contain sialylated glycoconjugates[2]. This observation raises many important questions on the biosynthetic pathway and regulation of sialic acid metabolism and its distribution and functions in plants. Currently, our laboratory is involved in studying function and regulation of genes and gene-products involved in sialic acid metabolism. A metabolic engineering approach is also being taken to enhance the level of sialylation in plants. This research will help us understand the sialic acid metabolism in plants and eventually manipulate sialylation in plants to produce glycoconjugates with desired glycan motifs. 1. Schauer, R. (2000). Achievements and challenges of sialic acid research. Glycoconjugate Journal 17: 485-499. 2. Shah, M., Fujiyama, K., Flynn R. C., Joshi, L. (2003). Sialylated endogenous glycoconjugates in plant cells. Nature Biotechnology 21(12): 1470-1471.

(24) **Free *N*-Glycans in Developing Plant Cells: Structural Features, Putative Function, and Related Enzymes**

Yoshinobu KIMURA.

Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University, Japan.

It has been reported that two kinds of free *N*-glycans, high-mannose type and plant complex type structures, occur in developing or growing plant cells. Although it has been postulated that such free *N*-glycans may play a critical role in plant cell development or growth, the potential function(s) still remain to be understood. As a part of study to elucidate the putative physiological function(s) of such free *N*-glycans in plant cells, we have revealed the structural feature of the free *N*-glycans and characterized several plant endo-β-*N*-acetylglucosaminidases and one peptide:*N*-glycanase (PNGase), which are responsible for the release of *N*-glycans.

All high-mannose type structures (Man5-9GlcNAc1) purified from hypocotyls of seedlings (pea, soybean, pumpkin), bamboo shoots, developing Ginkgo seeds, and some cultured cells (rice and tobacco cells) had a common core structure; Man α 1-6(Man α1-3)Man α1-6(Man α1-3)Man β1-4GlcNAc, suggesting the high-mannose type glycans had been released by the endoglycosidase. On the contrary, the plant complex type free *N*-glycans always had the chitobiosyl unit, suggesting that this type *N*-glycan had been derived by the glycoamidase. The concentration of high-mannose type free *N*-glycans (a few nano moles in gram fresh weight tissue) was more than 20 times higher than that of plant complex type free glycans.

The several plant endo-β-acetylglucosaminidases purified so far have a molecular weight of about 64 kDa, and show the optimum activity at weak acidic region (pH 6.0). The endoglycosidases could hydrolyze various high-mannose type *N*-glycans bearing α-1,2-mannosyl residue(s) at comparable rate. On the other hand, the relative reaction rates for Man5GlcNAc2 were 25-30% of those for Man6GlcNAc2, suggesting that the enzyme would have a specific subsite to recognize the α-1,2-mannosyl residue. Subcellular fractionation analysis using pumpkin cotyledons showed that the plant endoglycosidase resides and functions in the cytosol near the ER.

Although the amino acid sequences of some peptides purified from a lysylendopeptidase digest of the rice endo-β-GlcNAc-ase showed a homology with a putative Arabidopsis endo-β-GlcNAc-ase gene product, some sequences were not found in the deduced amino acid sequence, indicating that the plant endoglycosidase would have diversity in the genes encoded. Furthermore, recently, we have purified and characterized a Ginkgo α-mannosidase, which is activated by cobalt ion and prefers the high-mannose type free *N*-glycans occurring in plant cells to those having the *N*-acetylchitobiose unit as substrates, indicating that the mannosidase might be involved in the degradation of high-mannose type free *N*-glycans in the plant cell.

Concerning the physiological function of free *N*-glycans, we have recently reported that the plant complex type *N*-glycans bearing β-1,2-xylosyl and α-1,3-fucosyl residues could reduce a production of IL-4 from pollinosis patient T-cells stimulated by a pollen glycoallergen, indicating that the plant complex type *N*-glycans can be a candidate of glyco-drugs for a pollinosis therapy. [Reference] Kimura, Y., *Trends in Glycosci. Glycotech.,* 12, 103-112 (2000).

(25) **Biosynthesis and Functions of Glycosaminoglycans in Caenorhabditis Elegans**

Hiroshi Kitagawa.

Department of Biochemistry, Kobe Pharmaceutical University, Higashinada-ku, Kobe 658-8558,

Japan and CREST, JST, Kawaguchi, Saitama 332-0012, Japan..

Glycosaminoglycans (GAGs) such as heparan sulfate (HS) and chondroitin sulfate (CS) have been implicated in numerous pathophysiological phenomena of vertebrates and invertebrates. The critical roles of GAGs, especially HS, in developmental processes involving the signaling of morphogens such as Wingless and Hedgehog proteins, as well as of fibroblast growth factor, in *Drosophila* have recently become evident. The biosynthesis of GAGs is initiated by the addition of Xyl to specific serine residues in the core protein, followed by the sequential addition of two Gal residues and a GlcA residue, forming the tetrasaccharide linkage structure GlcAß1-3Galß1-3Galß1-4Xylß1-*O*-Ser. Then, HS polymerization with alternating GlcNAc and GlcA is achieved by an enzyme complex composed of EXT1 and EXT2 in the *EXT* gene family (1), which were first identified as causative genes of a genetic bone disorder, hereditary multiple exostoses. Alternatively, chondroitin polymerization with alternating GalNAc and GlcA takes place on the linkage tetrasaccharide by the action of a complex consisting of chondroitin synthase (ChSy) (2) and chondroitin polymerizing factor (ChPF), a unique protein factor required for the polymerization (3). So far, the functionally redundant, multiple glycosyltransferases involved in GAG biosynthesis have been cloned. This redundancy makes it difficult to investigate the biosynthetic mechanism and functions of GAGs by gene knockout or characterization of individual glycosyltransferases. To investigate the biosynthetic mechanism and functions of GAGs *in vivo*, we have been using lower organisms such as *Caenorhabditis elegans*, because they are predicted to have few glycosyltransferases and a simple mechanism for production of the GAG chains. In fact, only two homologous genes, *rib-1* and *rib-2*, of the mammalian *EXT* genes (4), a *ChSy* ortholog (*cChSy* or *sqv-5*) (5) and a *ChPF* ortholog (*pfc-1* or *PAR2.4*) (6) were identified in the *C. elegans* genome. In addition, *C. elegans* produces HS and non-sulfated chondroitin but not CS. Interestingly, recent RNA interference experiments of *cChSy* gene revealed that non-sulfated chondroitin is indispensable in the morphogenesis and cell division of *C. elegans* (5). In this lecture, recent advances in the study of the biosyntheis and functions of glycosaminoglycans in *C. elegans* will be presented. (1) Kim, B.-T., Kitagawa, H., Tanaka, J., Tamura, J., and Sugahara, K. (2003) *J. Biol. Chem.* 278, 41618-41623. (2) Kitagawa, H., Uyama, T., and Sugahara, K. (2001) *J. Biol. Chem.* 276, 38721-3872. (3) Kitagawa, H., Izumikawa, T., Uyama, T., and Sugahara, K. (2003) *J. Biol. Chem.* 278, 23666-23671. (4) Kitagawa, H., Egusa, N., Tamura, J., Kusche-Gullberg, M., and Sugahara, K. (2001) *J. Biol. Chem.* 276, 4834-4838. (5) Muzuguchi, S., Uyama, T., Kitagawa, H., Nomura, K. H., Dejima, K., Gengyo-Ando, K., Mitani, S., Sugahara, K., and Nomura, K. (2003) *Nature* 423, 443-448. (6) Izumikawa, T., Kitagawa, H., Muzuguchi, S., Nomura, K. H., Nomura, K, Tamura, J., Gengyo-Ando, K., Mitani, S., and Sugahara, K. (2004) Submitted.

(26) **Biosynthesis and Function of the Plant Cell Wall Polysaccharide Pectin: Identification of a Proposed Galacturonosyltransferase Gene Family in Arabidopsis thaliana and Effect of Pectin on Human Prostate Cancer Cells**

Debra Mohnen[1], Jason D. Sterling[1], Kerry L. Hosmer[1], Melanie Atmodjo[1], Michael G. Hahn[1], V.S. Kumar Kolli[1], Crystal L. Jackson[1] and M. Vijay Kumar[2]

[1] Complex Carbohydrate Research Center, The University of Georgia, Athens, GA 30602,
[2] Medical College of Georgia and VA Medical Center, Augusta, GA 30912. .

Pectin is a complex family of plant cell wall polysaccharides comprising ~35% of the primary wall of all higher plants except the grass family, where it is ~10% of the wall (1). Pectins have multiple roles in plant growth, development and disease resistance; are gelling and stabilizing agents in the food industry; and are beneficial dietary components, nutraceuticals, and potential pharmaceuticals with multiple positive effects on human health. Pectin is the most structurally complicated polysaccharide in the plant wall, consisting of ~55%-70% homogalacturonan (HGA), 20-35%, rhamnogalacturonan-I (RG-I) and 10-19% of the substituted galacturonan rhamnogalacturonan II (RG-II). Based on the known structure of pectin, at least 14 enzyme activities are required to synthesize the activated sugar substrates and 58 glycosyl-, methyl- and acetyl-transferases are required to synthesize the pectic polysaccharides (2). We previously identified and characterized an α-1,4-galacturonosyltransferase (GalAT) in tobacco, pea, radish and Arabidopsis that transfers GalA from UDP-GalA onto endogenous pectin acceptors and showed that the HGA:GalAT is a membrane bound glycosyltransferase (3) with its catalytic site located in the Golgi lumen (4). Detergent-solubilized GalAT and membrane-bound GalAT in permeabilized membranes transfer GalA onto the non-reducing end (5) of HGA oligosaccharide (oligogalacturonide) acceptors of DP>9 in an apparent non-processive fashion (6,7). The solubilized Arabidopsis GalAT was partially purified, the most purified column fractions treated with trypsin, and the released peptides analyzed by tandem mass spectrometry. Comparison of the recovered amino acid sequences with the Arabidopsis DNA/Protein database identified two putative glycosyltransferases in the GalAT-containing fractions. Heterologous expression of one of these genes in mammalian cells resulted in low levels of GalAT activity in the transiently transfected cells. This Arabidopsis gene has been named (GALAT1) and the GalAT it encodes is named galacturonosyltransferase I (GALAT1). A putative GalAT gene family of 25 genes that group into different clades has been identified in Arabidopsis based on sequence similarity to GALAT1. Progress in characterizing GALAT1 and the other members of the GalAT Gene Family will be presented. To test the hypothesis that the reported ability of pectin to inhibit cancer growth and metastasis is due, at least in part, to the induction of apoptosis, the effects of several pectin preparations on human prostate cancer cells was determined. Specific pectin preparations were found to induce apoptosis in both androgen-responsive (LNCaP) and androgen-independent (LNCaP C4-2) human prostate cancer cells in vitro. The results of structure/function studies of the pectin that induces apoptosis will be presented. Funded by NRI competitive USDA awards 98-35304-6772 and 2001-35318-11111, NSF Award MCB-0313509, and DOE-funded center grant DE-FG05-93-ER20097, and the Georgia Cancer Coalition. (1) Ridley et al., (2001) Phytochemistry 57:929-967, 2001. (2) Mohnen (2002) Biosynthesis of pectins. In: Pectins and their Manipulation, edited by Seymour, G.B. and Knox, J.P.Oxford:Blackwell Publishing and CRC Press p. 52-98. (3) Doong et al., (1995) Plant Physiol. 109:141-152. (4) Sterling et al., (2001) Plant Physiol. 127:360-371. (5) Scheller et al., Planta 207:512-517, 1999. (6) Doong and Mohnen (1998) Plant J. 13:363-374, 1998. (7) Guillaumie et al. (2003) Carbohydr.Res. 338:1951-1960.

(27) *Leishmania* Require Sphingolipids (SLs) for Acidocalcisome Biogenesis and Infectivity, and Salvage SLs from the Mammalian Host which are Remodeled into Inositolphosphoceramide (IPC)

Kai Zhang[1], Fong-Fu Hsu[2], David A. Scott[1,3], Roberto Docampo[3], John Turk[2] and Stephen M. Beverley[1]
[1] Dept. of Molecular Microbiology, Washington University School of Medicine, St. Louis MO 63105 USA, [2] Dept. of Medicine, Washington University School of Medicine, St. Louis MO 63105 USA, [3] Dept. of Pathobiology and Center for Zoonoses Research, University of Illinois at Urbana-Champaign, Urbana, Illinois 61802, USA.Medicine.

Sphingolipids (SLs) play essential roles in signal transduction and membrane dynamics in most eukaryotes, but in the trypanosomatid protozoan *Leishmania major* their roles diverge significantly. Previously we showed that null mutants (*spt2*-) lacking serine palmitoyltransferase (SPT) activity or detectable SLs grew well and retained lipid rafts while replicating *in vitro*, but experienced catastrophic defects in membrane trafficking upon entry into stationary phase, and failed to differentiate to the infective metacyclic form. *spt2*- parasites were compromised in their ability to establish infections in cultured murine macrophages, although they retained the ability to enter macrophages silently and inhibit activation even while being destroyed. Surprisingly, after a short delay, rapidly progressive lesions appeared in mice, and amastigotes purified from these lesions were fully virulent in macrophage and mouse infections. While it is known that *Leishmania* spp take up SLs from the host, mass spectrometric analysis of *spt2*- amastigotes revealed the presence of parasite-specific inositol phosphorylceramides (IPCs). IPCs were found at levels exceeding that of the abundant free glycosylphosphatidyl lipids (GIPLs) which characterize this infective parasite stage. Inhibitor studies suggest that salvage occurs at the level of complex sphingolipids, arguing that parasites may carry out 'headgroup remodeling'. *spt2*- promastigotes exhibited an intriguing defect involving 'empty' acidocalcisomes (ACs), which we attribute to a probable origin of this organelle from the lysosomal/multivesicular body biogenesis pathway. However, ACs in *spt2*- amastigotes appeared quantitatively and morphologically normal. These data suggest that SLs salvaged by intracellular amastigotes could play key roles in AC biogenesis and parasite survival in the mammalian host.

(28) Newly Discovered Neutral Glycosphingolipids in Aureobasidin A-resistant *Zygomycetes*

Kazuhiro Aoki[1], Ryosuke Uchiyama[1], Suguru Yamauchi[1], Takane Katayama[1], Saki Itonori[2], Mutsumi Sugita[2], Noriyasu Hada[3], Junko Hada[3], Tadahiro Takeda[3], Hidehiko Kumagai[1] and Kenji Yamamoto[1]
[1] Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan, [2] Faculty of Liberal Arts and Education, Shiga University, Otsu 520-0862, Japan, [3] Kyoritsu University of Pharmacy, Minato-ku, Tokyo 105-8512, Japan.

Sphingolipids are essential membrane component of both mammalian and fungal cells. Because sphingolipid synthesis is essential for the growth and viability of fungi, it is likely that a blocking of the synthesis would efficiently inhibit cell growth. Therefore, the enzymes catalyzing the synthesis of inositol-containing sphingolipids that are present in fungi but absent in humans have been focused as targets for antifungal agents. Aureobasidin A is well known and widely used as an antifungal agent for *Eumycetes* including yeasts and fungi. It exhibits strong fungicidal activity against many pathogenic fungi, including *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. This antifungal agent inhibits inositol phosphorylceramide (IPC) synthase in fungal cells. The inhibition of this enzyme causes the depletion of essential sphingolipids in the fungal cells. Because it is recognized that all fungi have this enzyme, Aureobasidin A is potentially a broad spectrum antifungal. During studies on glycosphingolipids (GSLs) of fungi, we found for the first time that *Zygomycetes* species showed resisitance to Aureobasidin A. Therefore, we analyzed the GSLs in *Zygomycetes* species to investigate the Aureobasidin A resistant mechanism. At first, GSLs of fungi were separated into neutral, acidic, and zwitterionic fractions by ion-exchange column chromatography based on their polarities. The GSLs of all *Zygomycetes* species were recovered in the neutral fraction but not in the acidic and zwitterionic fractions. In general, acidic GSLs are found in all fungal cells and have been known as inositol phosphate-containing sphingolipid. However, the *Zygomycetes* species do not have inositol phosphate-containing sphingolipids. This seems to be the reason that Aureobasidin A is not effective for *Zygomycetes*. We found a novel family of neutral GSLs in these fungi and isolated these GSLs from *Mucor hiemalis*, which is a typical *Zygomycetes* species. Their structures were completely determined by compositional sugar, fatty acid, and sphingoid analyses, methylation analysis, matrix-assisted laser desorption ionization time-of-flight/mass spectrometry, and ^{1}H-NMR spectroscopy. They were as follows: Galβ1-6Galβ1-1Cer (CDS), Galα1-6Galβ1-6Galβ1-1Cer (CTS), Galα1-6Galα1-6Galβ1-6Galβ1-1Cer (CTeS), and Galα1-6 Galα1-6 Galα1-6Galβ1-6Galβ1-1Cer (CPS). The ceramide moieties of these GSLs consisit of 24:0, 25:0, and 26:0 2-hydroxy acids as major fatty acids and 4-hydroxyoctadecasphinganine (phytosphingosine) as the sole sphingoid. Our results indicate that the biosynthetic pathway for GSLs in *Zygomycetes* is significantly different from those in other fungi and suggest that any inhibitor of this pathway may be effective for mucormycosis, which is a serious pathogenic disease for humans. Our finding may facilitate the development of new antifungal agents for Mucolales.

(29) *N*-linked Protein Glycosylation in *Campylobacter jejuni*

Lorna A. Millar[1], Nicolas Cadotte[1], Luc Tessier[1], Laura Fiori[1], John Kelly[1], Harold C. Jarrell[1], Jean-Robert Brisson[1], Brenda Allan[2] and Christine M. Szymanski[1]

[1] National Research Council of Canada, Ottawa, Canada, [2] Vaccine and Infectious Disease Organization, Saskatoon, Canada.

Recently, we described the first report of a bacterial *N*-linked protein glycosylation pathway in the common foodborne pathogen, *Campylobacter jejuni*. We have shown that the heptasaccharide: GalNAc-α-1,4-GalNAc-α-1,4-[Glc-β-1,3-]GalNAc-α-1,4-GalNAc-α-1,4-GalNAc-α-1,3-Bac-(Asn-X-Ser/Thr), where Bac is 2,4-diacetamido-2,4,6-trideoxy-D-glucopyranose and the Pgl enzymes (for protein glycosylation) required for the biosynthesis of this glycan are highly conserved. Therefore, this pathway is an attractive therapeutic target for the reduction of *C. jejuni* in food animals. Similar bacterial *N*-linked glycosylation systems have recently been identified in *Wolinella succinogenes* and *Desulfovibrio desulfuricans*. We are interested in gaining a better understanding of the bacterial *N*-linked glycosylation pathway and its biological role using *C. jejuni* as our model system. In order to determine if the complete oligosaccharide must be synthesized before the glycan can be transferred to protein, we have analyzed mutants in the *pgl* locus for the presence of the heptasaccharide using high resolution magic angle spinning (HR-MAS) NMR. To allow unambiguous detection of the glycan by HR-MAS NMR, the *pgl* mutants were created in a capsule-minus background. Where reduced or aberrant glycosylation may have led to signals below NMR detection limits, proteins from select mutants (including the glycoprotein Peb3) were further examined by mass spectrometry. Glycopeptides were analyzed by ESI-TOF MS followed by MS-MS sequencing for protein identification and glycan characterization. Together the data indicate that the glycan is transferred as a block with relaxed specificity for the branched glucose. Furthermore, an acetyltransferase homologue from an alternate biosynthetic pathway can compensate for heptasaccharide biosynthesis in a *pglD*- background while mutation of *pglG* had no effect on protein glycosylation under the conditions examined. In *C. jejuni*, disruption of the general *N*-linked glycosylation pathway has been shown to affect host cell interactions, probably through the inactivation of multiple proteins. In order to investigate the therapeutic potential of this pathway, we examined the effect of *pgl* mutations on *C. jejuni* colonization in the chicken commensal host. As expected, *pglB* and *pglH* mutants showed complete loss of colonization while the *pglG* mutant showed similar levels of colonization compared to wildtype. Interestingly, the *pglI* mutant exhibited reduced levels of colonization although its glycoproteins only lack the glucose branch. Further studies are aimed at understanding the role and regulation of the *C. jejuni N*-linked glycosylation pathway.

(30) **UDP-sugar Pyrophosphorylase with Broad Substrate Specificity Towards Various Monosaccharide 1-phosphates from Pea Sprouts**

Toshihisa Kotake[1], Daisuke Yamaguchi[1], Hiroshi Ohzono[1], Sachiko Hojo[1], Tomoyuki Konishi[1],
Satoshi Kaneko[2], Hide-ki Ishida[3] and Yoichi Tsumuraya[1]
[1] Department of Biochemistry and Molecular Biology, Faculty of Science, Saitama University, 255 Shimo-okubo, Sakura-ku, Saitama 338-8570, Japan, [2] Biological Function Division, National Food Research Institute, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8642, Japan, [3] the Noguchi Institute, 1-8-1 Kaga, Itabashi-ku, Tokyo 173-0003, Japan.

Activated nucleotide sugars that serve as glycosyl donors for polysaccharides and of the sugar moieties of glycolipids and glycoproteins in higher plants are generated through *de novo* pathway, in which various UDP- and GDP-sugars are produced through sequential interconversions from UDP-Glc and GDP-Man as the starting substrates. In the salvage pathway, an alternative route to synthesize nucleotide sugars, free monosaccharides released during the degradation of polysaccharides and glycoconjugates are first phosphorylated by the action of monosaccharide kinases, then converted into nucleotide sugars by the action of pyrophosphorylases in the presence of respective nucleotide triphosphates as co-substrates. Pyrophosphorylases to generate UDP-pentoses (UDP-L-Ara and UDP-Xyl) from respective pentose 1-phosphates in plants have neither purified nor cloned so far.

We looked for activity of UDP-L-Ara pyrophosphorylase in plant sources and found the activity, together with that of UDP-Glc pyrophosphorylase, in a crude enzyme preparation of pea sprouts. The enzyme designated *PsUSP* (*Pisum sativum* UDP-sugar pyrophosphorylase) was purified from the crude preparation by ammonium sulfate fractionation and several subsequent chromatographic operations. At the final purification stage, the enzyme was purified approximately 1,200-fold. The apparent molecular mass of the purified PsUSP was 67,000 Da on SDS-PAGE. The enzyme catalyzed the formation of UDP-Glc, UDP-Gal, UDP-GlcA, UDP-L-Ara, and UDP-Xyl from respective monosaccharide 1-phosphates in the presence of UTP as a co-substrate, indicating that the enzyme has broad substrate specificity towards monosaccharide 1-phosphates. Maximum activity of the enzyme occurred at pH 6.5 to 7.5, and at 45˚C in the presence of 2 mM Mg^{2+}. The apparent K_m values for Glc 1-phosphate and L-Ara 1-phosphate were 0.34 and 0.96 mM, respectively.

PsUSP cDNA was cloned by reverse transcriptase-PCR. *PsUSP* appears to encode a protein with molecular mass of 66,040 Da (600 amino acids) and possesses a uridine-binding site which has also been found in a human UDP-HexNAc pyrophosphorylase (AGX1). The sequence of PsUSP shared low but significant similarity (15%) with AGX1. Tertiary structure prediction with the 3D-PSSM program showed that the structure of PsUSP is closely related to AGX1, although GlcNAc 1-phosphate served as poor substrate for PsUSP. Phylogenetic analysis revealed that PsUSP can be categorized in a group together with homologues from *Arabidopsis* and rice, which is distinct from UDP-Glc and UDP-HexNAc pyrophosphorylase groups. Recombinant PsUSP expressed in *Escherichia coli* catalyzed the formation of UDP-sugars from monosaccharide 1-phosphates and UTP with efficiency similar to that of the native enzyme. These results indicate that the enzyme is a novel type of UDP-sugar pyrophosphorylase, which catalyzes the formation of various UDP-sugars at the end of salvage pathway in higher plants.

(31) **Roles of N-Glycans on Integrin-Mediated Signaling**

Jianguo Gu and Naoyuki Taniguchi
Dept. Biochem., the 21st Century COE program, Osaka Univ. Med. Sch.
B1, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.

Integrins consisting of α and β subunits, are cell surface receptors for extracellular matrix (ECM) molecules that are present in all animals. Integrins are involved in several fundamental cell biological processes. First, and most prominently, they mediate the adhesion of cells to their substrates by providing a physical link between the ECM and the cytoskeleton. Second, integrins can act as signaling receptors that relay information concerning the substrate to the interior of the cell (outside-in signaling), which can, in turn, be interpreted as growth, differentiation, or survival signals. Although integrin-mediated adhesion is based on its ligand, the strength of this binding is modulated by various factors including the status of the glycosylation of integrin. In fact, these cell surface integrins are all major carriers of N-glycans. Changes in the N-glycan structures of these integrins can also affect cell-cell and cell-ECM interactions, thereby affecting cell adhesion, migration and tumor malignancy. To characterize effects of a specific N-glycan on integrin functions as well as its association with other molecules, we have performed a manipulation of glycogenes using genetic and biochemical approaches including the induction of gene expression and gene knockout. The introduction of the bisecting GlcNAc results in the suppression of further processing and the elongation of N-glycans catalyzed by other glycosyltransferases, since they are not able to use the bisected oligosaccharide as a substrate. Thus, GnT-III is generally regarded to be a key glycosyltransferase in N-glycan biosynthetic pathways. We have found that the metastatic capabilities of B16 mouse melanoma cells are down regulated by the introduction of the GnT-III gene. This anti-metastatic effect has been, in part, attributed to the effect of GnT-III on an increase in E-cadherin-mediated homotypic adhesion and the suppression of the phosphorylation of the E-cadherin-β-catenin complex on the cell-cell adhesion. Recently, we found that the modification of the N-glycans of integrin by GnT-III inhibits its ligand binding ability, subsequently leading to the down-regulation of cell spreading and migration as well as integrin-mediated signaling. Accordingly, the overexpression of GnT-III inhibits tumor metastasis by at least two mechanisms: an enhancement of cell-cell adhesion and a down-regulation of cell-ECM adhesion. We also found that the modification of core fucose plays important roles in integrin-mediated cell migration. Considering the integrins contain multiple potential N-linked glycosylation sites on each of α and β subunit, so it is essentially important to completely characterize N-glycans on these potential sites for developing a better understanding of the functional roles of glycosylation in regulating physiological cellular signaling and pathological processes such as inflammation, infection, cancer, and cancer metastasis as well.

(32) **Carbohydrate-Carbohydrate Interaction-Dependent Neutrophil Chemotaxis and Phagocytosis**

Kazuhisa Iwabuchi[1,2,4], Tadashi Sato[1,3], Toshihide Kobayashi[4], Kumiko Ishii[4] and Hideoki Ogawa[1]
[1] Institute for Environmental and Gender-specific Medicine, Juntendo University Graduate School of Medicine, [2] Laboratory of Biochemistry, Juntendo University School of Health Care and Nursing, [3] Department of

Respiratory Medicine, Juntendo University Graduate School of Medicine, [4] Sphingolipid Functions Laboratory, Frontier Research System, RIKEN.

Neutrophils are important professional phagocytic cells that provide the host with a first line of defense against acute bacterial and fungal infections. In mature neutrophils, more than 70% of GSLs are lactosylceramide (LacCer, CDw17). Interestingly, both gram-negative and -positive bacteria and pathogenic fungi can bind to LacCer. Therefore, LacCer has been speculated to be participated in bactericidal action of neutrophils. LacCer forms lipid rafts coupled with a Src family kinase Lyn, named as LacCer-enriched glycosignaling domain (GSD) on neutrophil plasma membrane, and neutrophils generate superoxide anion through LacCer-enriched GSD. We show here that pathogenic fungi-derived β-glucan specifically binds to LacCer through carbohydrate-carbohydrate interaction to induce neutrophil chemotaxis. To provide insights about the dynamic mechanisms of LacCer-enriched microdomains in neutrophil functions, we observed molecular dynamics of LacCer on neutrophil plasma membrane during β-glucan-induced migration and phagocytosis using time-lapse confocal microscopy. Candida albicans-derived soluble β-glucan CSBG, which has β-(1,6) long side chains, dose-dependently induced neutrophil chemotaxis. In contrast, mushroom-β-glucan SCG and GRN, which have β-(1,6) mono glycoside side chains, did not significantly induce neutrophil migration. Interestingly, CSBG-induced neutrophil migration was completely blocked by LacCer. Moreover, anti-LacCer antibody T5A7 dose-dependently induced neutrophil migration. Taken together, these results suggest that fungi-derived β- (1,3) glucans induce neutrophil chemotaxis and phagocytosis via the carbohydrate-carbohydrate interactions between LacCer and β-glucan.

(33) **Significance of Cytoplasmic Prolyl Hydroxylation and Complex Glycosylation in the Cellular Slime Mold *Dictyostelium***

Christopher M. West[1], Hanke van der Wel[1], Slim Sassi[2], Eric Gaucher[3] and Altan Ercan[1]

[1] Dept. of Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104 USA, [2] Dept. of Anatomy & Cell Biology, University of Florida College of Medicine, Gainesville, FL, 32610 USA, [3] Foundation for Applied Molecular Evolution, Gainesville, FL 32601 USA.

The small nucleocytoplasmic glycoprotein Skp1 is a target for a novel pathway of prolyl hydroxylation and glycosylation resulting in the formation of a complex hydroxyproline-linked pentasaccharide in the cellular slime mold Dictyostelium discoideum. Based on enzyme protein purification and sequencing, genes encoding a polypeptide (pp) αGlcNAcT and a bifunctional βGalT/αFucT have been cloned that can form Skp1's core trisaccharide, Fucα1,2Galβ1,3GlcNAcα- (equivalent to the type I blood group H antigen). A requirement for the βGalT/αFucT for Skp1 glycosylation in vivo has been shown by gene disruption. These genes have counterparts in the secretory pathway which are required for mucin-type O-glycosylation, polysaccharide biosynthesis, and normal spore coat function. To identify the predicted prolyl 4-hydroxylase (P4H) gene, a comparative bioinformatics approach was taken to search for the sequence in Dictyostelium's genome most likely to encode this enzyme. Of five predicted cytoplasmic P4H sequences, one was most similar to putative P4H sequences from an oomycete and a diatom each joined in frame with putative cytoplasmic pp αGlcNAcT sequences. In addition, this Dictyostelium sequence was most related to a recently characterized class of oxygen-dependent P4Hs that target the transcriptional factor subunit hypoxia-inducing factor-1α and other proteins for ubiquitin (Ub)-mediated degradation in animals. Recombinant P4H-1 was found to exhibit the predicted activity toward Skp1-Pro143, and P4H-1 was required for Skp1 hydroxylation in vivo based on absence of Skp1 glycosylation in a P4H-1-null mutant generated by gene disruption. P4H-1-null cells exhibited a developmental arrest phenotype suggesting an inability to execute culmination, the process in which slug cells rearrange to form a fruiting body with differentiated stalk and spore cells. Arrest did not occur in βGalT/αFucT-null cells suggesting that the phenotype did not result from failure to construct the full length pentasaccharide normally found on Skp1. Related developmental arrest phenotypes have been observed in Dictyostelium strains possessing null alleles for cullin-A and FbxA, proteins known to engage with Skp1 to form the SCF class of E3 Ub-ligases involved in turnover of regulatory proteins. Cytoplasmic P4H-1 may act on Skp1 to regulate oxygen-dependent turnover of a key developmental regulatory factor, which may be important for ensuring that culmination does not occur before slugs emerge from the soil onto aerobic surfaces that favor later spore dispersal. (Supported by NIH R01-GM-37539)

(34) **Mapping and Quantifying O-Glycosylation Sites and Proteins Using a Mass-Spectrometry Based Comparative Proteomic Tool**

Lance Wells, Dan Sherling, Jae-Min Lim, Bryan Woosley, Ron Orlando and Carl Bergmann

University of Georgia, Complex Carbohydrate Research Center, Athens, GA USA.

Taking advantage of several of the recent advancements in mass spectrometry, we demonstrate the mapping and quantifying of O-glycosylation. We have previously developed and demonstrated the use of a beta-elimination/Michael addition with dithiothreitol (BEMAD) strategy for mapping sites of O-linked beta-N-acetylglucosamine (O-GlcNAc) modification on nuclear and cytosolic proteins (Mol. Cell. Proteomics 1:791). This approach allows for the replacement of the labile O-linked sugar with a 'DTT tag' that can be used for enrichment and as a signature for the site of modification upon collison-induced dissociation (CID) fragmentation sequencing of the peptide by tandem mass spectrometry. Here we show the evolution of the BEMAD methodology into a comparative proteomic tool for mapping and quantifying sites of O-glycosylation as well as proteins with as little as 100 attomoles of peptide. We show that the BEMAD approach serves as a simple, inexpensive alternative to the ICAT (isotope-coded affinity tag) methodology for relative quantification of proteins by the use of D6-DTT and the susceptibility of alkylated cysteines, by carboxyamidomethylation with iodoacetamide, to beta-elimination. We demonstrate that BEMAD can be used for mapping O-glycosylation sites besides that of O-GlcNAc. As well, we prove that relative changes in O-glycosylation can be quantified using D0- and D6-DTT. Further, we show the usefulness of neutral-loss experiments in a linear ion-trap mass spectrometer to map sites of O-glycosylation. Also, we illustrate the usefulness of electron-capture dissociation (ECD) fragmentation in a Fourier transform-linear ion trap (LTQ-FT, Finnigan) for mapping sites of O-glycosylation. The power of combining these approaches is exemplified by mapping multiple sites of O-glycosylation from biologically-derived, in vivo modified proteins.

(35) **O-GlcNAc A Mediator of Cellular Function: Characterizing a Family of O-GlcNAc Binding Proteins**

Natasha E Zachara and Gerald W Hart

Department of Biological Chemistry, The Johns Hopkins University School of Medicine.

O-linked β-N-acetylglucosamine is an essential and dynamic post-translational modification of Serine and Threonine residues of nuclear and cytoplasmic proteins of metazoans. Modulation of O-GlcNAc levels has profound effects on cells, alluding to the importance of this post-translational modification in regulating cellular function. The precise mechanism(s) by which O-GlcNAc alters proteins function remain elusive. One way O-GlcNAc may alter protein function is by mediating protein-protein interactions. Many intracellular lectins have previously been described, including several proteins which bind O-GlcNAc notably HSP70, CBP35 and CBP70. Here we show that HSP70 and two proteins previously shown to bind Hyaluronan, cdc37 and HABP1, also bind GlcNAc. In preliminary studies, we show that HAPB1, HSP70, and cdc37 bind BSA-GlcNAc, but not BSA or BSA-GalNAc in both far-western and elisa experiments. HABP1 is known to bind the essential splicing factor ASF/SF2 that we have shown to be glycosylated in the cytoplasm. In far western experiments, HABP1 binding to ASF/SF2 can be blocked by 1M GlcNAc or pre-incubation of the blot with sWGA. We are currently determining which other intracellular proteins are bound by these proteins in an O-GlcNAc dependent manner. Isolation of an O-GlcNAc binding motif, analogous to phospho-amino acid binding domains such as SH2 domains, will greatly enhance our understanding of the role of O-GlcNAc in cellular processes. Supported by HD13563 and CA42486 to GWH, and the NHLBI NIH contract No. N01-HV-28180. Under a licensing agreement between Covance Research Products and The Johns Hopkins University, Dr. Hart receives a share of royalty received by the university on sales of the CTD 110.6 antibody.

(36) **Molecular Pathogenesis of Type 2 Diabetes via Insulin Signaling in Membrane Microdomains: Involvement of Ganglioside GM3**

Jinichi Inokuchi.

Department of Biomembrane and Biofunctional Chemistry and 2Core Research for Evaluational Science and Technology program (CREST), Japan Science and Technology Corporation (JST), Graduate School of Pharmaceutical Sciences, Frontier Research Center for Post-Genomic

Science and Technology, Hokkaido University, Kita 21-Nishi 11, Kita-ku, Sapporo 001-0021, Japan..

We will establish the molecular pathogenesis of type 2 diabetes and insulin resistance by testing our working hypothesis "Life cycle-related diseases, such as type 2 diabetes, are a membrane microdomain syndrome caused by aberrant expression of glycosphingolipids (GSLs)." Based on these efforts, we will explore new therapeutic strategies for type 2 diabetes, such as microdomain ortho-signaling therapy. Membrane microdomains (lipid rafts) are now recognized as critical for proper compartmentalization of insulin signaling, but their role in the pathogenesis of insulin resistance has not been investigated. Detergent-resistant membrane microdomains (DRMs), isolated in the low density fractions, are highly enriched in cholesterol, glycosphingolipids and various signaling molecules. TNF induces insulin resistance in type 2 diabetes, but its mechanism of action is not fully understood. We have found a selective increase in the acidic glycosphingolipid ganglioside GM3 in 3T3-L1 adipocytes treated with TNF, suggesting a specific function for GM3 (J. Biol. Chem., 277, 3085-3092, 2002). In the DRMs from TNF-treated 3T3-L1 adipocytes, GM3 levels were doubled, compared to results in normal adipocytes. Additionally, insulin receptor (IR) accumulations in the DRMs were diminished, while caveolin and flotillin levels were unchanged. Furthermore, insulin-dependent IR internalization and intracellular movement of the IR substrate 1(IRS-1) were both greatly suppressed in the treated cells, leading to an uncoupling of IR-IRS-1 signaling. GM3 depletion was able to counteract the TNF-induced inhibitions of IR internalization and accumulation into DRMs. Together, these findings provide compelling evidence that in insulin resistance the insulin metabolic signaling defect can be attributed to a loss of IRs in the microdomains due to an accumulation of GM3.

(37) **Signal Transduction of Proteoglycan-Type Protein Tyrosine Phosphatase, PTPzeta and Development of the Brain**

Nobuaki Maeda.

Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu, Tokyo 183-8526, Japan.

PTPzeta is a brain-specific receptor-type protein tyrosine phosphatase, which is synthesized as a chondroitin sulfate (CS) proteoglycan. The extracellular region of this receptor is secreted as a soluble CS proteoglycan, phosphacan. These transmembrane and secreted forms are generated by alternative splicing. PTPzeta uses a heparin-binding growth factor pleiotrophin (PTN) as one of the major ligand, in which CS portion plays an essential role in ligand binding. The expressions of PTPzeta and PTN are dynamically regulated during development of cerebellum. While PTPzeta is expressed by cerebellar Purkinje cells and Bergmann glia, PTN is produced by Bergmann glia. Purkinje cells and the processes of Bergmann glia are closely associated, and it has been supposed that this cell-cell interaction plays important roles in the morphogenesis of Purkinje cells. Thus we hypothesized that PTPzeta-PTN signaling is involved in the morphogenesis of Purkinje cells. Using an organotypic slice culture system, we observed that an aberrant morphology of Purkinje cell dendrites such as multiple and disoriented primary dendrites was induced by addition of function-blocking antibodies against PTPzeta, chondroitinase ABC (Chase ABC) digestion, and addition of exogenous CS chains. The effects of CS was structure-dependent, and GlcA(2S)1-3GalNAc(6S) disaccharide unit (D unit)-rich CS strongly induced aberrant morphogenesis of Purkinje cells. The extracellular space between Purkinje cells and the processes of Bergmann glia are enriched with D unit-rich CS, and D unit-rich CS strongly inhibits the binding of PTN to PTPzeta, suggesting that this oversulfated structure plays important roles in the PTPzeta-PTN signaling. In order to examine the significance of CS structures in the PTN signaling, we purified phosphacan from postnatal day 7 (P7) and P12 rat cerebral cortex (PG-P7 and PG-P12, respectively) and from P20 rat whole brain (PG-P20). The chondroitin sulfate chains of these preparations displayed immunologically and compositionally different structures. Analysis of the Chase ABC digestion products revealed that GlcA1-3GalNAc(4S) disaccharide unit (A unit) was the major component in these preparations and that PG-P20 contained 1.3% D unit, which was not detected in PG-P7 and PG-P12. Interaction analysis using BIAcore system indicated that PG-P20 had ~5-fold stronger affinity for PTN (Kd = 0.14 nM) than PG-P7 and PG-P12, although all these preparations showed similar low affinity binding to PTN after Chase ABC digestion (Kd = 1.4 ~ 1.6 nM). These results suggest that variation of CS plays important roles in the regulation of signal transduction in the brain. References (1) Tanaka, M., Maeda, N., Noda, M., Marumouchi, T. (2003). A chondroitin sulfate proteoglycan PTPzeta/RPTPβ regulates the morphogenesis of Purkinje cell dendrites in the developing cerebellum. J. Neurosci. 23, 2804-2814. (2) Maeda, N., He, Y., Yajima, Y., Mikami, T., Sugahara, K., Yabe, T. (2003). Heterogeneity of the chondroitin sulfate portion of phosphacan/6B4 proteoglycan regulates its binding affinity for pleiotrophin/heparin binding growth-associated molecule. J. Biol. Chem. 278, 35805-35811.

(38) **Cell surface Localization of Heparanase on Macrophages Regulates Degradation of Extracellular Matrix Heparan Sulfate**

Norihiko Sasaki[1], Nobuaki Higashi[1], Tomohiro Taka[1], Motowo Nakajima[2] and Tatsuro Irimura[1]

[1] Laboratory of Cancer Biology and Molecular Immunology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, [2] Tsukuba Research Institute, Novartis Pharma.

Degradation of extracellular matrix components including heparan sulfate proteoglycans (HSPGs) is a critical step for extravasation through vascular basement membrane. Heparanase, the heparan sulfate-specific endo-β-glucuronidase, has previously been shown to be a key enzyme in melanoma invasion. Transmigration of immune cells such as peripheral blood monocytes through basement membrane should also require HSPG degradation and this process should be strictly regulated to avoid excess cellular infiltration and tissue damage. However, mechanisms that regulate the HSPG degradation have not been well elucidated in the immune system. We examined a potential regulatory mechanism of heparanase in HSPG degradation and transmigration through basement membranes in monocyte trafficking.

Human promonocytic leukemia U937 and THP-1 cells treated with PMA are differentiated to be macrophage-like cells. The cells were shown to degrade ^{35}S-sulfated HSPG in endothelial extracellular matrix into fragments of an approximate molecular mass of 5 kDa. This was not found with untreated cells. The gene expression levels of heparanase or the enzyme activity of the amount of cell lysates were no different between untreated and treated cells, indicating that transcriptional regulation is unrelated to the induced HSPG degradation of the cells. Immunocytochemical staining with anti-heparanase mAb revealed pericellular distribution of heparanase in PMA-treated cells but not in untreated cells, suggesting that recruitment of heparanase to the plasma membrane seems to be crucial for the HSPG degradation. This was supported by the fact that microtubule disruption by nocodazole suppressed both pericellular distribution of heparanase and HSPG degradation. When the PMA-treated cells were allowed to adhere, cell surface heparanase capped into a restricted area on the cells. Addition of a chemoattractant fMLP induced polarization of the PMA-treated cells and heparanase redistribution at the leading edge of migration. Therefore a major regulatory process of heparanase activity in the cells seems to be surface expression and capping of the enzyme. Addition of the anti-heparanase mAb significantly inhibited enzymatic activity and transmigration of the PMA-treated cells, suggesting that the cell surface redistribution of heparanase is involved in monocyte extravasation through basement membranes.

To prove that the cell surface expression and capping of heparanase occur in nontransformed monocytes, CD14+ peripheral blood monocytes that had been allowed to adhere were stained with anti-heparanase mAb under nonpermeabilized condition. The unique spacial distribution of heparanase also appeared in normal human monocytes, *i.e.*, a large portion of monocytes (68.7 Å} 7.1%) was stained with the mAb, 34.3 Å} 10.3% of which showed capping formation. As heparanase seems to be involved in degradation of HSPG and extravasation, the enzyme expression is a marker to define monocyte subpopulation that represents high transmigratory capacity. Identification of the monocyte subpopulation and functional significance of heparanase and heparan sulfate in the subpopulation are currently studied.

Reference: N. Sasaki *et al.* (2004) *J. Immunol.* 172: 3830-3835.

(39) **A Ligand and Carbohydrate Engagement (LACE) Assay Detects Changes in Heparan Sulfate Expression During Mouse Development**

Alan C. Rapraeger, Benjamin L. Allen, Ravishankar Ramaswamy and Xinping Yue

Department of Pathology and Laboratory Medicine, University of Wisconsin-Madison, Madison, WI 53706.

Heparan sulfate glycosaminoglycans bind numerous developmental morphogens, including Wnts, hedgehog, BMPs, chordin, and FGF family members. These proteins regulate critical developmental processes and their

activity in these processes depends upon exquisite binding to heparan sulfate glycosaminoglycans that are expressed throughout the developing organs. A major question, therefore, is whether the synthesis of specific sulfation patterns or domains within heparan sulfate changes during development in a spatiotemporal manner and whether these changes govern the ability of the heparan sulfate to regulate these factors. We have focused on FGF family members to examine this question. Because it is currently not possible to isolate and sequence discrete heparan sulfate chains from individual developing tissues, we have examined heparan sulfate binding activity in situ using binding probes. Using a ligand and carbohydrate engagement (LACE) assay, we directly visualize the assembly of FGFs and the extracellular domains of their receptors (FR) expressed as recombinant proteins with endogenous heparan sulfate expressed in mouse embryonic tissues during development. Focusing on FGF1 and FGF8b interactions with FR2c and FR3c, we find that global changes in heparan sulfate expression in mouse embryos during development serve to regulate FGF and FR complex assembly. Furthermore, distinct heparan sulfate requirements are identified for the assembly and signaling by each FGF and FR pair. We also find that FGF4, a reported angiogenic growth factor, shows differential binding to the heparan sulfate in blood vessels, including both the endothelial cells and surrounding smooth muscle cells; this differential binding suggests that the expression of heparan sulfate surrounding veins and arteries may be quite different. Overall, our findings suggest that changes in heparan sulfate act as a critical temporal regulators of growth factor and morphogen signaling during embryogenesis. (Supported by NIH grant GM48850)

(40) **Deficiency of Heparan Sulfate N-Deacetylase/N-Sulfotransferase-1 in Endothelium Impairs Selectin and Chemokine Mediated Neutrophil Trafficking**

Lianchun Wang[1], Mark M. Fuster[2], Nissi Varki[3], P. Sriramarao[4] and Jeffrey D. Esko[1]

[1] Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, La Jolla, CA 92093, [2] Department of Medicine, University of California, San Diego, La Jolla, CA 92093, [3] Department of Pathology, University of California, San Diego, La Jolla, CA 92093, [4] Division of Vascular Biology, La Jolla Institute for Molecular Medicine, San Diego, CA 92121.

Endothelial heparan sulfate has been hypothesized to play a role in inflammation by binding to proteins involved in leukocyte trafficking. Here we examined this issue in vivo using a mouse strain lacking the biosynthetic enzyme, GlcNAc N-deacetylase/N-sulfotransferase-1 (NDST1). Mice bearing a loxP-flanked allele of NDST1 (NDST1f/f) were crossed to tie2Cre mice, which expressed the bacterial Cre recombinase in the endothelium and leukocytes. Loss of NDST1 resulted in a 70% reduction in glucosamine N-sulfate content and dramatic reduction in FGF binding to endothelial cells in vivo. NDST1f/fTie2Cre+ mice showed impaired responses in various inflammatory models (thioglycollate-induced peritonitis, delayed type hypersensitivity reactions, and chemokine induced migration in air pouches). The velocity of neutrophil rolling was greater on activated endothelial cells derived from the mutant, which correlated with weaker interactions of recombinant L-selectin with the cells. Additionally, neutrophil arrest was less efficient. These effects resulted from alterations in heparan sulfate in the endothelium rather than on leukocytes based on bone marrow transplantation experiments. Our findings provide direct evidence supporting a role for endothelial heparan sulfate in inflammation in vivo and are consistent with reduced binding and transcytosis of chemokines to the luminal surface of the endothelial cells.

(41) ***N*-Acetylglucosamine-6-*O*-Sulfotransferase-1 Deficiency Causes Loss of Keratan Sulfate in the Developing Brain and Injured Brain**

Kenji Kadomatsu[1], Haoqian Zhang[1], Kenji Uchimura[2] and Takashi Muramatsu[1]

[1] Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan,
[2] Department of Anatomy, Program in Immunology, University of California, San Francisco, CA94143, U.S.A..

N-Acetylglucosamine-6-*O*-sulfotransferases (GlcNAc6STs) catalyze the transfer of sulfate to position 6 of *N*-acetylglucosamine (GlcNAc) residues. So far, five GlcNAc6STs have been identified in humans. 6-Sulfated GlcNAc is present in glycoproteins and keratan sulfate (KS). Recent studies have shown that GlcNAc6ST-5 is specifically expressed in the cornea and involved in synthesis of corneal KS. However, the GlcNAc6ST that is essential for synthesis of KS in other tissues has been still unidentified. We have observed that GlcNAc6ST-1 is expressed in specific regions of the developing mouse brain. To investigate the physiological function of GlcNAc6ST-1, we produced GlcNAc6ST-1-deficient mice, and have recently reported that GlcNAc6ST-1 is involved in lymphocyte homing to lymph nodes and Peyer's patches. We found that the expression of KS in the developing thalamus and cerebral cortex was totally abolished in GlcNAc6ST-1-deficient mice. The KS immunoreactivity in the cerebral cortex of wild-type mice was in the area projected from the thalamus, suggesting that keratan sulfate is involved in construction of neural network. The expression of KS in the vertebrae did not alter in the deficient mice, indicating that still other GlcNAc6ST participates in formation of vertebrae in embryonic mice. The spatiotemporal expression of KS following brain injury was also examined. A striking increase in KS immunoreactivity was observed in the parenchyma surrounding the lesion in wild-type mice, but not in the deficient mice. Our data indicate that GlcNAc6ST-1 is the enzyme that produces KS in the brain. We are currently analyzing the possible function of KS in the developing brain and injured brain using GlcNAc6ST-1-deficient mice.

(42) **Molecular and Cellular Functions of Proteoglycans in Tissue Assembly and Morphogenesis**

Scott B. Selleck.
Developmental Biology Center, and Depts. of Pediatrics and Genetics, Cell Biology & Development,
University of Minnesota, Minneapolis, MN 55455.

My laboratory is interested in how heparan proteoglycans (HSPGs) function to control tissue morphogenesis. We use a number of model organisms and systems to explore how these fascinating molecules function at the molecular and cellular level. I will present findings from Drosphila and zebrafish that document the critical role of HSPGs in general, and glypicans in particular, in controlling cellular responses to growth factors. In addition, recent findings demonstrate that glypicans play critical roles in regulating the distribution of morphogens, including BMPs and Wnt family members. Our studies include direct analysis of in vivo function of these molecules using molecular and genetic tools, as well as biophysical measures of glypican-growth factor interactions. Current work is directed at understanding the role glypicans play in assembly of the nervous system.

(43) **Functional Glycomics Using *Drosophila* RNAi system**

Shoko Nishihara[1,2], Mika Hino[2,3], Hideki Yoshida[1,2], Norihiko Sasaki[1,2], Satoshi Goto[2,3], Hidenao Toyoda[2,4] and Ryu Ueda[2,5]

[1] Laboratory of Cell Biology, Department of Bioinformatics, Faculty of Engineering, Soka University, [2] Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Agency (JST), [3] Genetic Networks Research Group, Mitsubishi Kagaku Institute of Life Science, [4] Department of Bio-analytical Chemistry, Graduate School of Pharmaceutical Sciences, Chiba University, [5] Invertebrate Genetics Laboratory, National Institute of Genetics.

Glycan structures on proteins and lipids are developmentally regulated and play an important role for cell-cell recognition. The glycosylation is performed in the Golgi apparatus by glycosyltransferases, which transfer sugars from sugar-nucleotides to acceptor substrates. Sugar-nucleotide transporters supply a variety of sugar-nucleotides, which are synthesized in the cytosol, as donor substrates of glycosyltransferases in the Golgi apparatus. The elucidation of the biological role of glycan is one of the most important issues to be resolved following the genome project. *Drosophila melanogaster* is well established as a model for genetic analysis. Our molecular evolutionary study showed that a prototype of glycosyltransferases was conserved between mammals and *Drosophila*. RNA interference (RNAi) is becoming an efficient reverse genetic tool for studying gene function in model organisms. For analyses of the basic physiological functions of glycans, we established the *Drosophila* inducible RNAi system and applied it to glycosyltransferases and sugar-nucleotide transporters.

If the silencing of each gene was induced ubiquitously in the fly, many RNAi mutant flies showed lethality to prove essential roles of glycans in development. The tissue specific RNAi induced various malformations in the flies, by which genes were classified into some groups. These clusters might reflect the biosynthetic pathway of glycans. We will present a new approach to the comprehensive analysis of glycan function in development.

(44) **Neurotic, a GDP-fucose O-fucosyltransferase, Regulates Notch Turnover and Endocytic Transportation Independently of Its Enzymatic Activity.**

Takeshi Sasamura[1,2], Hiroyuki O Ishikawa[3], Syunsuke Higashi[2], Maiko Kanai[1,2], Shiho Nakao[2], Nobuo Sasaki[2], Tomonori Ayukawa[2], Toshiro Aigaki[4], Katsuhisa Noda[5], Naoyuki Taniguchi[5] and Kenji Matsuno[1,2,3]
[1] PRESTO, Japan Science and Technology Agency, [2] Department of Biological Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan, [3] Genome and Drug Research Center, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan, [4] Department of Biological Sciences, Tokyo Metropolitan University, 1-1 Minami-osawa, Hachioji-shi, Tokyo 192-0397, Japan, [5] Department of Biochemistry, Division of Molecular Therapy Science, Osaka University
Graduate School of Medicine, 1-7 Yamada-oka, Suita, Osaka 565-0871, Japan.

In multi-cellular organisms, cell-cell interaction plays essential roles in their development. The Notch signaling pathway is an evolutionarily conserved signaling mechanism that regulates a very broad spectrum of cell-specification through local cell-cell communication. In the Drosophila embryo, defects in Notch signaling results in neurohyperplasia, generally referred to as the neurogenic phenotype. We have identified a novel maternal neurogenic gene, designated as neurotic (*nti*). We showed that *nti* encodes an essential component of Notch signaling in various contexts of Drosophila development. *nti* encodes a protein highly homologous to mammalian GDP-fucose O-fucosyltransferase, which is an enzyme that adds a fucose to serine or threonine residues in the consensus sequence CXXGGS/TC (X is any amino acids) between the second and third conserved cysteines of epidermal growth factor-like (EGF) repeats. Interestingly, Fringe (Fng), a modulator of Notch signaling, adds N-acetylglucosamine (GlcNAc) onto the O-fucose moieties of Notch and its ligands. Modification of Notch by Fng, and subsequent glycosylation of Notch, modulates the binding of Delta and Serrate to Notch. These reports and our results suggest that nti enables Notch to respond to its ligands by adding O-linked fucose to the Notch EGF repeats. We also showed that Nti is essential for the physical interaction between Notch and Delta in Drosophila cultured cells. In addition to its requirement for Notch-ligand interactions, we found that Nti regulates turnover and trafficking of the Notch protein. While Notch was mostly detected in endocytic vesicles in the wild-type cells, we found that Notch was abnormally accumulated in intracellular vesicles before reaching to the plasma membrane in *nti* mutant cells. These vesicles did not coincide with markers for, Golgi, endoplasmic reticulum (ER), endosome, nor lysosome, suggesting they may be a uncharacterised vesicle compartments in Drosophila. Importantly, this novel activity of Nti was independent of its O-fucosyltransferase activity, because in mutants of GDP-mannose 4,6-dehydratase (GMD) ortholog gene, which failed to produce GDP-fucose, Notch was delivered to the plasma membrane normally, although Notch activity was abolished in these mutants as expected. Using live tissue labeling with an antibody against the extracellular domain of Notch, we showed that some portion of Notch was still able to reach to the plasma membrane in *nti* mutant cells. However, in these cells, Notch at the apical membrane localized abnormally, and endocytic vesicles containing Notch was largely eliminated. Taken together, our results indicate that Nti plays essential roles for both transportation of Notch to the plasmamembrane and subsequent endocytosis of Notch. This idea is also supported by our results that turnover of Notch is reduced in *nti* mutant cells and accelerated by an overexpression of Nti. Finally, we found that overexpression of Nti, which promoted turnover of Notch, reduced that Notch signaling activity. A mutant Nti lacking an ER-retention signal, which did not localize to ER anymore, was more potent to suppress the endogenous Notch signaling. Together, we propose that Nti may be involved in negative regulation of Notch via its novel and enzymatic activity-independent functions that accelerate turnover rate of Notch.

(45) **Action of Protein O-fucosyltransferase 1 within the Endoplasmic Reticulum in Maturation of the Notch Receptor**

Tetsuya Okajima, Aiguo Xu, Liang Lei and Kenneth D. Irvine
Piscataway NJ 08854 USA.

Abstract not available.

(46) **Glycomic Analysis of Mouse Embryonic Stem Cell Differentiation**

Steve Dalton[1], Kelley Moremen[4], Alison Nairn[4], Gerardo Alvarez-Manilla[4], Alfred Merrill[2], Jin-Kyu Lee[4], Intaek Lee[4], Karen Abbott[4], Rick Matthews[3] and Michael Pierce[4]
[1] Bresagen, Inc., Athens, GA 30602, [2] School of Biology, Georgia Institute of Technology, Atlanta, GA 30332, [3] Department of Neurobiology, Yale University School of Medicine, New Haven, CT 06520, [4] the Integrated Resource Center for Biomedical Glycomics, Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602.

The program of glycoprotein and glycolipid glycosylation clearly changes during embryonic development and cell differentiation, yet a coherent understanding of even the early stages of this program is lacking. The development of technologies to investigate the details of the glycosylation of glycoproteins and glycolipids in embryonic stem cells (ES) and their differentiated lineages offers an opportunity to characterize the glycome of ES cells and how it changes during the differentiation of ES cells to neuronal precursor cells. These technologies include quantitation of glycogene expression patterns, fractionation of glycopeptides followed by analysis of glycans and peptides by LC/MS, analysis of sphingolipids and glycosphingolipids by LC/MS, and synthesis of data by an informatics component. Withdrawal of the growth factor, LIF, from ES cultures, for example, causes reversible differentiation into ectoderm primitive layer (EPL) cells that are still pluripotent and correspond to the first step of ectodermal differentiation. An initial investigation of glycan expression revealed ES but not EPL cells were bound by the lectin DBA. The majority of DBA reactivity was found to correspond to the expression of the glycan terminating in Gal(alpha1,3), known as Forssman antigen, and the expression of this glycan was lost during differentiation to EPL cells; reversion to ES cells by adding LIF resulted in re-expression of DBA reactivity. Interestingly, this glycan is expressed specifically on a single glycoprotein, CD9, a member of the tetraspanin family whose function can regulate differentiation. The changes in the expression of additional glycans and the glycosyltransferases that synthesize them during differentiation will also be presented. The Integrated Resource Center for Biomedical Glycomics is supported by the National Center for Research Resources at NIH and involves collaboration with additional investigators including the Functional Glycomics consortium.

(47) **Regulation of Notch signaling by *Drosophila heparan sulfate 3-O sulfotransferase***

Keisuke Kamimura[1,2], John M. Rhodes[3], Melissa McNeely[4], Deepak Shukla[4], Koji Kimata[2], Patricia G. Spear[4,5], Nicholas W. Shworak[3] and Hiroshi Nakato[1]
[1] Department of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN 55455, USA, [2] Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-1195, Japan, [3] Section of Cardiology and Angiogenesis Research Center, Department of Medicine, Dartmouth Medical School, Dartmouth Hitchcock Medical Center, Lebanon, NH 03756, USA , [4] Department of Microbiology-Immunology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611, USA, [5] Current address: Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, Illinois 60612, USA.

Heparan sulfate (HS) regulates the activity of various ligands and is involved in molecular recognition events on the cell surface and in the extracellular matrix. Specific binding of HS to different ligand proteins depends on the sulfation pattern of HS. The first and still the most notable example showing the importance of sequence-specific sulfation in HS-protein interactions comes from analyses of 3-*O* sulfated HS. In humans, there are at least two distinct forms of 3-*O* sulfated HS. One form of 3-*O* sulfated HS (HS^{AT+}) generated by HS 3-*O* sulfotransferases-1 (HS3ST-1), binds to antithrombin (AT), resulting in inhibition of blood coagulation. However, a recent report showed that *Hs3st1*$^{-/-}$ knockout mice do not exhibit an obvious procoagulant phenotype, implying other physiological roles for 3-*O* sulfation. A different form of 3-*O* sulfated HS (HS^{gD+}) generated by HS3ST-3 isoforms, interacts with a viral coat protein, glycoprotein D (gD), to promote entry of the herpes simplex virus-1 (HSV-1) into host cells. However, an endogenous ligand for this HS sequence is not known. Interestingly, sequences homologous to mammalian *HS3ST* genes are found in the genome of *Drosophila melanogaster* and *Caenorhabditis elegans* despite the very dissimilar blood coagulation and viral entry systems. The existence of *HS3ST* homologues in these organisms suggests that 3-*O* sulfation has a role in evolutionarily conserved processes, such as developmental pathways. To examine the in vivo importance of a specific sulfation of HS for the interaction with binding proteins, we studied the function of 3-*O* sulfated HS during *Drosophila* development. We isolated cDNA clones for two *Drosophila HS 3-O sulfotransferases* (*Hs3st-A* and *B*), and characterized their structures, expression, and functions. Homology comparison of human and *Drosophila* HS3STs indicated that Hs3st-A was most similar to HS3ST-1 and 5, which both generate HS^{AT+}. Conversely,

Hs3st-B was related to the remaining enzymes, many of which preferentially generate HS^{gD+}. Transfectional analyses showed that Hs3st-B, like HS3ST-3 isoforms, mediated cellular entry of HSV-1. These results suggested that Hs3sts exhibited distinct yet phylogenetically conserved sequence specificities. We used the transgenic RNAi technique to analyze the in vivo function of *Hs3st-A* and *B*. Transgenic RNAi of either *Hs3st-A* or *B* caused lethality, showing that both genes are critically required for viability. We found that reduction of *Hs3st-B* function by transgenic RNAi compromised Notch (N) signaling, producing neurogenic phenotypes. *Hs3st-B* genetically interacts with known neurogenic genes such as *N*, *Delta* (*Dl*), *kuzbanian*, *deltex*, and *scabrous*. Furthermore, *Hs3st-B* transgenic RNAi eliminated expression of *N* target genes, indicating that *Hs3st-B* functions in N signaling. Epistatic analysis showed that *Hs3st-B* acts downstream of *Dl* expression and upstream of N activation. In addition, levels of N protein were markedly reduced by *Hs3st-B* RNAi. These findings show that Hs3st-B is a novel regulator of the N signaling pathway and controls pattern formation. We also observed that the *Hs3st-B* RNAi affects the ditribution of Golgi apparatus and the number and size of endosomal/lysosomal compartments, suggesting the possibility that 3-*O* sulfated HS affects intracellular trafficking of N protein.

(48) **Dolichol-Cycle of Protein N-Glycosylation is Critical for Angiogenesis**

Juan A. Martinez[1], Anarda N. Gonzalez[2], Aurymar Sanchez[3], Krishna Baksi[3] and Dipak K. Banerjee[1]

[1] Department of Biochemistry, School of Medicine, Medical Sciences Campus, University of Puerto Rico, San Juan, PR 00936-5067. USA, [2] Department of Pathology, School of Medicine, Medical Sciences Campus, University of Puerto Rico, San Juan, PR 00936-5067. USA, [3] Department of Anatomy and Cell Biology, School of Medicine, Universidad Central del Caribe, Bayamon, PR 00960-6032. USA.

Angiogenesis is the formation of new capillaries from pre-existing vasculature by migration and proliferation of capillary endothelial cells. It is essential for normal growth and development, and is also a 'key step' in tumor growth and invasion. Angiogenesis from preexisting vasculature occurs in stages that orchestrate a network of cooperative interactions, a significant component of which is endothelial cell migration, capillary budding, establishment of capillary loops, and neovascular remodeling. A large number of cytokines accelerate the process of angiogenesis, help facilitate a switch to the angiogenic phenotype, and enhance glycosylation of proteins. Attachment of N-glycans to the protein core gives security and increases the functional diversity of asparagine-linked (N-linked) glycoproteins. The process begins in the endoplasmic reticulum (ER) upon initiation with the assembly of $Glc_3Man_9GlcNAc_2$-PP-Dolichol (lipid-linked oligosaccharide, LLO). We have been studying the regulation of LLO biosynthesis in a non-transformed capillary endothelial cell line and its relationship to angiogenesis. Treatment of these cells with a GlcNAc-1 phosphate transferase inhibitor, tunicamycin (an 84-dalton glucosamine-containing pyrimidine nucleoside) resulted in down-regulation of LLO biosynthesis, the expression of cell surface N-glycans as well as the inhibition of cell growth due to cell cycle arrest in G1. Light microscopic analysis indicated cell shrinkage, loss of membrane contact with neighboring cells, apparent compaction of nuclei showing condensed pyknotic appearance and membrane fragmentation. Scanning electron microscopy evidenced the presence of surface blebbing in cells treated with tunicamycin. The response was time- and concentration-dependent and could not be reversed either by a protein synthesis inhibitor (cycloheximide), or by the fibroblast growth factor-2 (FGF-2). Kinetic studies with protein expression by Western Blotting exhibited a reduced expression of G1 cyclins, cdk2 and bcl-2. High-level expression of p53-regulated $p21^{WAF1/Cip1}$ further confirmed the cell growth arrest in G1. Examination of the functional status of the ER in tunicamycin treated cells exhibited higher expression of two ER chaperons, Bip/GRP-78 and GRP-94, and supported the presence of an 'ER stress', which ultimately led to the induction of apoptosis due to *unfolded protein response*. Flow cytometry, DNA laddering, annexin V binding, pathological staining of the thick sections as well as analysis of selective apoptotic markers not only confirmed our above observations but also led us to conclude that capillary endothelial cell death in the presence of tunicamycin was indeed due to apoptosis. Supported by the Department of Defense grant DAMD17-03-1-0754.

(49) **Protein O-mannosylation and Its Pathology**

Tamao Endo.

Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, Itabashi, Tokyo 173-0015, Japan.

Protein *O*-mannosylation is rare protein modification and is present in a limited number of glycoproteins of brain, nerve, and muscle. One of the best-known *O*-mannosyl-modified glycoproteins is alpha-dystroglycan, which is a central component of the dystrophin-glycoprotein complex isolated from skeletal muscle membranes. Walker-Warburg syndrome (WWS) is autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormalities and lissencephaly. The *POMT1* gene is responsible for WWS. POMT1 has protein *O*-mannosyltransferase activity, but only when it is co-expressed with another homologue POMT2. All 7 mutations found in the *POMT1* gene of patients with WWS lead to great reduction of protein *O*-mannosyltransferase activity. These findings suggest that protein *O*-mannosylation of alpha-dystroglycan is important in muscle and brain development.

(50) **Expanding Perspectives on Congenital Disorders of Glycosylation**

Hudson H. Freeze, Xiaohua Wu, Ognian Bohorov, Erik Eklund and Liangwu Sun

The Burnham Institute, 10901 N. Torrey Pines Rd. La Jolla CA, 92037 USA.

The 17 Congenital Disorders of Glycosylation (CDG) fall into two groups. Defects in the biosynthesis and transfer of the lipid-linked oligosaccharide precursor (LLO) chain to nascent proteins in the ER lumen define CDG-I disorders. The 12 known disorders (CDG-Ia to CDG-IL) account for only about one third of the potential candidate genes. The great majority of proven patients (~400) are classified CDG-Ia (phosphomannomutase-deficient) with <100 patients falling into the known Type I defects. The second group (CDG-II) broadly covers all other genes involved in oligosaccharide biosynthesis and processing. Five types are known (CDG-IIa through CDG-IIe), but there are scores of potential candidate genes. Proven Type II defects currently account for <20 cases. Electrospray mass spectrometry of serum transferrin (Tf) distinguishes Types I from II based on the absence of entire chains (I) vs. monosaccharides (II). Serum oligosaccharide profiling and detailed analysis provides clues to possible Type II defects, but none of the analytical methods provides a gene-specific diagnosis. In the last two years, our laboratory found that a minority of patients are CDG-Ia (20%) or have other known types (25%), while half have novel Type I or II defects. Many of the Type II disorders also show deficiencies in O-linked oligosaccharide biosynthesis ruling out N-linked oligosaccharide-specific processing defects. We found that one set of severely affected patients was deficient in multiple glycosyltransferases and nucleotide sugar transporters due to a mutation in COG7. This protein is a subunit of the cytoplasmic COG complex that associates with the Golgi. The COG complex is involved in shuttling proteins between the ER and Golgi and within the Golgi. Fluorescence recovery after photobleaching (FRAP) of Golgi-localized GFP-tagged ST3Gal-I showed that trafficking is much slower than controls. Transfection with wild-type COG7 normalizes glycosylation, glycosyltransferase activities, transporters, and ST3Gal-1 trafficking. Several other CDG-IIx patients also show similar abnormal glycosylation, partial glycosytransferase and nucleotide sugar transporter deficiencies, and prolonged FRAP. These findings suggest that the abnormal glycosylation likely results from altered trafficking of multiple proteins, rather than defects within a single oligosaccharide biosynthetic pathway. This novel perspective greatly expands the spectrum of candidate genes that could cause CDG-II. It encourages the use of abnormal glycosylation as a simple and general diagnostic tool, but the new perspective also challenges traditional approaches to identify the defects in CDG patients. (Supported by NIH RO1DK55615)

(51) **From Graft Failure to Graft-Versus-Host Disease: the Central Role of Glycans in Allogeneic Bone Marrow Transplantation**

Robert Sackstein, MD,PhD.

Harvard Institutes of Medicine, 77 Ave. Louis Pasteur, Room 671, Boston, MA, 02115.

Bone marrow transplantation (also known as 'hematopoietic stem cell transplantation') is curative therapy for aplastic anemia and a variety of hematologic malignancies and genetic diseases. For this treatment to be successful, donor hematopoietic stem cells must first engraft in sufficient numbers to allow recovery of white blood cell counts within a few weeks post-transplant. Delayed engraftment or, worse yet, overt graft failure results in life-threatening infectious complications. However, following engraftment, another life-threatening complication can result from the expanding donor lymphocyte population launching an immune attack

against host tissues, an entity known as acute graft-versus-host disease (GVHD) that principally affects the skin, liver and gut. For several years, our laboratory has investigated the molecular basis of graft failure and of acute GVHD. Recent studies using human cells and tissues have revealed that both of these processes are driven by cell-specific and site-specific carbohydrate modifications. Importantly, the presentation of relevant glycan structures is temporally and spatially precise, exerting exquisite control on the biology of hematopoiesis and immune reactivity. Among the critical effectors of these processes are the human 'bone marrow homing receptor' HCELL (for Hematopoietic Cell E-/L-selectin Ligand) and extracellular matrix elements such as hyaluronic acid. HCELL is an N-linked sialofucosylated glycoform of CD44 expressed exclusively on the earliest human hematopoietic progenitor cells, and it is the most potent native human E-selectin ligand. In contrast to the typical pattern of inducible E-selectin expression, human bone marrow microvascular endothelial cells permanently express E-selectin, wherein the HCELL-E-selectin axis has a primary role in recruitment of stem cells into the marrow. In acute cutaneous GVHD, sialofucosylations of lymphocyte PSGL-1 rendering the 'skin homing receptor' CLA (for Cutaneous Lymphocyte Antigen) and specialized deposits of hyaluronic acid on the papillary dermal endothelium each occur and collectively promote migration of alloreactive lymphocytes into the skin, leading to tissue injury. An important goal of our research is to develop reagents to selectively modify expression and activity of pertinent carbohydrates to improve the clinical outcomes for patients undergoing bone marrow transplantation.

(52) **Carbohydrate Mimicry between Human Ganglioside GM1 and *Campylobacter jejuni*Lipo-oligosaccharide Causes Guillain-Barre Syndrome**

Nobuhiro Yuki.
Department of Neurology, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan.

Molecular mimicry between microbial and self components is postulated as the mechanism that accounts for the antigen and tissue specificity of immune responses in post-infectious autoimmune diseases. Little direct evidence exists, and research in this area has focused principally on T cell-mediated, anti-peptide responses, rather than on humoral responses to carbohydrate structures. Guillain-Barre syndrome, the most frequent cause of acute neuromuscular paralysis, occurs 1 to 2 weeks after various infections, particularly *Campylobacter jejuni* enteritis. Carbohydrate mimicry [Gal β1-3 GalNAc β1-4 (NeuAc α2-3) Gal β1-] between the bacterial lipo-oligosaccharide and human GM1 ganglioside is seen as having relevance to the pathogenesis of Guillain-Barre syndrome, and conclusive evidence is reported here. On sensitization with *C. jejuni* lipo-oligosaccharide, rabbits developed anti-GM1 IgG antibody and flaccid limb weakness. Paralyzed rabbits had pathological changes in their peripheral nerves identical to those present in Guillain-Barre syndrome. Immunization of mice with the lipo-oligosaccharide generated a monoclonal antibody that reacted with GM1 and bound to human peripheral nerves. The monoclonal antibody and anti-GM1 IgG from patients with Guillain-Barre syndrome did not induce paralysis, but blocked muscle action potentials in a muscle-spinal cord co-culture, indicative that anti-GM1 antibody can cause muscle weakness. These findings show that carbohydrate mimicry is an important cause of autoimmune neuropathy. (References. Yuki. Infectious origins of, and molecular mimicry in, Guillain-Barre and Fisher syndromes. Lancet Infect Dis 2001;1:29-37 : Yuki et al. Acute axonal polyneuropathy associated with anti-GM1 antibodies following *Campylobacter* enteritis. Neurology 1990;40:1900-1902 : Yuki et al. A bacterium lipopolysaccharide that elicits Guillain-Barre syndrome has a GM1 ganglioside-like structure. J Exp Med 1993;178:1771-1775 : Yuki et al. Animal model of axonal Guillain-Barre syndrome induced by sensitization with GM1 ganglioside. Ann Neurol 2001;49:712-720 : Yuki et al. Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipo-oligosaccharide causes Guillain-Barre syndrome. Proc Natl Acad Sci USA 2004 in press)

(53) **A Glucose Transporter N-Glycosylation Defect Promotes Type 2 Diabetes**

Kazuaki Ohtsubo[1], Shinji Takamatsu[2], Mari T. Minowa[3], Aruto Yoshida[4], Makoto Takeuchi[4] and Jamey D. Marth[1]
[1] Howard Hughes Medical Institute and Department of Cellular and Molecular Medicine, 9500 Gilman Drive, University of California San Diego, La Jolla, California, 92093, [2] Biomedical Imaging Research Center, University of Fukui, 23-3 Shimoaizuki, Matsuoka, Yoshida, Fukui, 910-1193 Japan, [3] Bioinformatics Center, Hitachi, Ltd., Life Science Group, 1-3-1 Minamidai, Kawagoe, Saitama 350-1165 Japan, [4] Central Laboratories for Key Technology, Kirin Brewery Co. Ltd., 1-13-5, Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan.

Glucose transport is an essential process in cellular metabolism and provokes insulin secretion by the pancreatic beta cell to reduce extracellular glucose concentrations. We have found that these physiologic events depend upon the Mgat4a-encoded and medial-Golgi resident GlcNAcT-IVa glycosyltransferase. Early in adulthood, mice lacking a functional Mgat4a gene and GlcNAcT-IVa activity become diabetic with impairments in glucose tolerance and insulin secretion. High triglyceride levels and liver damage are found, and insulin resistance evolves. These disease signs occur without pancreatic inflammation and in the presence of normal islet abundance and morphology. We find that disruption of glucose homeostasis and insulin secretion are due to the requirement of the Glut-2 glucose transporter for a tetra-antennary N-glycan structure produced with the GlcNAcT-IVa glycosyltransferase. Without a functioning Mgat4a gene, the defective Golgi-derived N-glycan branching pattern on Glut-2 reduces its cell surface half-life and expression by 10-fold in the presence of a normal Km for glucose. Lectin binding analyses of N-glycan structures on other pancreatic beta cell glycoproteins, such as the Insulin Receptor-alpha subunit, show that the same defect in glycosylation does not impair expression or stability of these other molecules. Pulse-chase analyses and co-localization studies using antibodies and cellular markers show increased internalization and degradation of Glut-2 in the absence of a functional Mgat4a gene. These findings indicate that N-glycosylation by GlcNAcT-IVa in the Golgi apparatus of pancreatic beta cells comprises a novel and specific mechanism that promotes cell-surface expression of the Glut-2 glucose transporter. A defect in this post-translational modification reduces pancreatic beta cell glucose entry, attenuates insulin secretion, and promotes the development of type 2 diabetes.

(54) **Induction of Lymphocyte Homing Receptors in the Human Gastric Mucosa Infected by *Helicobacter pylori***

Motohiro Kobayashi[1,2], Junya Mitoma[1], Tsutomu Katsuyama[3], Jun Nakayama[2] and Minoru Fukuda[1]
[1] Glycobiology Program, Cancer Research Center, The Burnham Institute, La Jolla, CA 92037, USA, [2] Department of Pathology, Shinshu University School of Medicine, Matsumoto 390-8621, Japan, [3] Department of Laboratory Medicine, Shinshu University School of Medicine, Matsumoto 390-8621, Japan.

Helicobacter pylori infects over half the world's population and is a leading cause of peptic ulcer and gastric cancer. The infection of *H. pylori* is usually confined to the surface mucous cell-derived mucin. On the other hand, our recent studies demonstrate that gland mucous cell-derived mucin, which contains alpha 1,4-GlcNAc-capped core 2-branched *O*-glycans, inhibits *H. pylori* growth and thus acts as a natural antibiotic against *H. pylori* infection (1). *H. pylori* infection results in chronic inflammation of the gastric mucosa, and progression of chronic inflammation leads to pyloric gland atrophy and intestinal metaplasia. However, how this chronic inflammation is induced or maintained is not well known. We analyzed randomly selected 144 biopsy specimens obtained from patients with chronic gastritis (including normal mucosa), and *H. pylori* infection was confirmed immunohistochemically in 85/123 (69.1%) samples with chronic gastritis. Even in samples failed to detect *H. pylori* immunohistochemically, intestinal metaplasia was observed in 23/37 (62.2%) cases, strongly suggest that *H. pylori* was once present. These combined indicate that 108/123 (87.8%) cases were regarded as *H. pylori*-associated chronic gastritis. This result is consistent with the fact that more than 90% of chronic gastritis in Japan are associated with *H. pylori* infection. In chronic inflammatory states of other systems, L-selectin and its ligands are implicated in lymphocyte recruitment in those diseases where lymphocyte homing receptors (peripheral lymph node addressin) are induced on high endothelial venule (HEV)-like vessels. Here, we show that chronic inflammation caused by *H. pylori* infection is highly correlated with *de novo* synthesis of lymphocyte homing receptors presented on HEV-like vessels. The number of HEV-like vessels dramatically increases as chronic inflammation progresses. We found that the lymphocyte homing receptors are bound by L-selectin-IgM chimeric protein, and decorated by NCC-ST-439 antibody, which recognizes both non-sulfated and 6-sulfated sialyl Lewis X on core 2 branched *O*-glycans, and MECA-79 antibody, which reacts with 6-sulfo *N*-acetyllactosamine on extended core 1 *O*-glycans (2). These results indicate that lymphocyte homing receptors on HEV-like vessels present in the gastric mucosa subsequent to *H. pylori* infection are similar to those on HEVs present in the secondary lymphoid organs, which are essential for

lymphocyte circulation. Moreover, eradication of *H. pylori* is associated with the disappearance of HEV-like vessels in the gastric mucosa. These results strongly suggest that lymphocyte homing receptors in HEV-like vessels play a critical role in lymphocyte recruitment during chronic inflammation induced by *H. pylori infection* (supported by NCI grants R37CA33000, P01CA71932, and Priority Area 14082201 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan).

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(55) MALDI and Electrospray MS Strategies for Glycomics and Glyco-Proteomics.

Anne Dell[1], Mark Sutton-Smith[1], David Goldberg[2], Maria Panico[1], Sara Chalabi[1], Nyet-Kui Wong[1], Paul Hitchen[1], Jihye Jang-Lee[1], Simon North[1], Victoria Ledger[1], Simon Parry[1], Stuart Haslam[1] and Howard Morris[1]
[1] Imperial College of Science, Technology and Medicine, Department of Biological Sciences, London SW7 2AZ, UK,
[2] Scripps-PARC Institute for Advanced Biomedical Sciences, Palo Alto, USA.

Ultra-high sensitivity mass spectrometric strategies for defining the primary structures of highly complex mixtures of glycoproteins are revolutionising structural glycobiology in the post-genomic era. MS strategies incorporating MALDI-MS and ES-MS/MS enable very complex mixtures from biological extracts and/or glycopolymer digests to be screened thereby revealing the types of glycans present and, importantly, providing clues to structures that are likely to be functionally important. We have devised MS strategies which enable the glycome of cells, tissues and organs to be examined and the glycoforms of individual glycoproteins to be identified. We are employing MALDI mapping and ES-MS/MS sequencing of permethylated N-and O-glycans in order to define the glycomes of wildtype and knockout mice. These methodologies have been adopted by the NIH Consortium for Functional Glycomics whose Analytical Core is carrying out high throughput analyses of mice and human tissues in order to provide a glycomics data resource for the glycobiology community. In our glycoproteomics studies we largely exploit nanospray and on-line nanoLC-ES-MS/MS technology. These methodologies have yielded important new information on the O-glycosylation of zona pellucida glycoproteins from normal and transgenic mice, and from a variety of novel bacterial glycoproteins. In order to make the above methodologies practical for high-throughput screening, reliable automatic methods of identifying peaks in MALDI and ES-MS/MS spectra must be devised. We are developing algorithms that automatically label ions in spectra of permethylated glycans with cartoons which represent the most plausible sequences consistent with the molecular ion masses, the types of glycans being analysed and the repertoire of fragment ions obtained by collisional activation. This work is supported by the BBSRC, the Wellcome Trust and the NIH. Anne Dell is a BBSRC Professorial Fellow. We are grateful to our collaborators and their teams whose projects underpin this abstract.

(56) A System for Rapid Identification of Oligosaccharide on Glycoprotein Using an Observational MS^n Spectral Library

Akihiko Kameyama[1], Norihiro Kikuchi[2], Shuuichi Nakaya[1,3], Hiromi Ito[1], Takashi Sato[1], Toshihide Shikanai[1,2], Yoriko Takahashi[2] and Hisashi Narimatsu[1]
[1] Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan,
[2] Mitsui Knowledge Industry Co., Ltd., Tokyo, Japan, [3] Shimadzu Corporation, Kyoto, Japan.

Glycosylation is the most wide-spread post-translational modification in eukaryotes, however the role of oligosaccharides attached to proteins has been studied little because of the lack of a sensitive and easy analytical method for oligosaccharide structures. Recently tandem mass spectrometric techniques have been revealing that oligosaccharides might have characteristic fragment patterns. These facts prompted us to develop a practical and systemized technology, aiming at the glycan structure analysis, which is based on a combination of MS^n spectral library and computer technology. We describe here a system for rapid and accurate identification, including the regio-, and stereo-chemistry, of the oligosaccharides of glycoproteins using MS alone. It is based on a comparison of the fragment patterns of MS^n spectra between the analyte and an observational spectral library which is built up by acquiring MS^n spectra of a large variety of structurally defined oligosaccharides. To build up the MS^n spectral library, the fragment patterns of each oligosaccharide, sample information such as the structure and labeling reagent, and experimental conditions (acquisition mode, matrix etc) were stored in a relational database. Oligosaccharide structures were described in the library as XML (extensible markup language) format we developed [1]. We have cloned and characterized many human glycogenes in the past several years [2]. Unlike in previous trials by others, our strategy utilizes a large variety of structurally defined oligosaccharides which can be obtained not only from natural sources but also by an effortless and rapid enzymatic preparation using a library of glycosyltransferases directed by the glycogenes which have been accumulated in our laboratory. To identify the oligosaccharides released from biological materials, i) a MS^2 spectrum of the sample is acquired, and its fragment pattern is sent to the search server. ii) The computer chooses several oligosaccharides in the library as candidates based on similarity with the fragment pattern of the sample. iii) The differences between the fragment patterns of MS^3 spectra of the candidates are evaluated by the computer, and the fragment ion with the largest difference in the library is selected as the "Next parent ion (priority 1)". iv) MS^3 spectrum of sample, only on the fragment ion suggested to be the "Next Parent Ion", is acquired. v) The similarity between the fragment pattern of the acquired MS^3 spectrum and corresponding profiles of the candidates are evaluated with the computer, and the candidate with the highest level of similarity to the sample is considered to be identical to the sample. Using this strategy we were able to identify the structure of N-linked oligosaccharides in transferrin and immunoglobulin G as examples. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO). 1. Kikuchi, N. et al, submitted to *Bioinformatics*
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(57) Application of Capillary Electrophoresis to Glycomics: High-Speed Screening of Carbohydrate Chains

Kazuaki Kakehi.
Kinki University, Faculty of Pharmaceutical Sciences, Kowakae 3-4-1, Higashi-Osaka, 577-8502, Japan.

Glycosylation is one of the most important post-translational events for proteins, and plays significant roles in various information traffics for intracellular and intercellular biological events in health and disease states. Thus, the interaction between proteins capable of binding glycoconjugates and carbohydrates is quite stimulating because carbohydrates are the mediator for the transmission of biological information. Many methods have been developed for the analysis of the interaction between carbohydrates and carbohydrate-binding proteins. Most of them such as surface plasmon resonance, fluorescence polarization and time-resolved fluorometry are based on the interaction between one protein and one carbohydrate. However, it is important to compare subtle difference in affinity when considering that highly heterogeneous carbohydrate chains are present in a glycoprotein.
We have developed a method for the analysis of a complex mixture of carbohydrates using capillary electrophoresis in a buffer containing a lectin of which binding specificity is well established. Comparison of migrations in the absence and presence of the lectin clearly indicates structure characteristics of carbohydrates. We have examined many commercially available lectins, and established a protocol available for the analysis of wide-range of complex oligosaccharides including both N- and O-linked carbohydrate chains. It should be noted that the method allows simultaneous determination of the interaction between carbohydrates and carbohydrate-binding proteins without prior isolation of the target carbohydrates. We successfully confirmed the presence of di- and tri-fucosylated oligosaccharides in alpha1-acid glycoprotein derived from serum samples of cancer patients.
When a mixture of oligosaccharides of well-established compositions is employed as a sample solution in capillary electrophoresis using a buffer containing an unknown carbohydrate-binding protein, we can easily confirm the presence of a carbohydrate-binding protein from the migrations of the mixture. As an example, a new lectin recognizing tri-antennary oligosaccharides in tulip bulbs will be given.
We will also show some topics on a method for high-speed releasing of carbohydrate chains from protein core.

(58) **Glyco-Informatics Prediction of Sugar-Protein Interactions Using Protein Data Bank**

Tsuyoshi Shirai and Clara Shionyu-Mitsuyama
Bioinformatics section, Biomolecular Engineering Research Institut, Furuedai 6-2-3, Suita, Osaka Japan.

Despite of the increasing significance of sugar molecules in biology, availability of bioinformatics techniques for sugars still stays far behind those for proteins or nucleic acids. Considering sugar chains have tertiary (3D) structures, like other biopolymers, that determine their functions, a development of method for predicting interaction and conformation of sugar chains on the bases of 3D information would take high priority. Because most sugar chains are synthesized by enzymes, and are recognized by lectins, Protein Data Bank (PDB) is the richest source of information to be used for predictions. PDB is growing rapidly, and it already contains a considerable amount of examples of sugar-protein interaction. However, the required information is not mined in the database in a ready-to-use fashion. To make the best use of PDB, sugar molecules need to be discriminated from many other molecules, and redundant data should be removed. And more importantly, the information should be updated regularly. We are currently developing a computer system that can automatically process the PDB into a database of sugar and sugar-binding protein molecules. As a tentative statistics, the recent PDB (2004.01) contained ~22,000 entries, and about 9% (~2,000) of them contained sugar moieties. After redundant protein structures were removed by the processing system, the number of entries reduced to ~1,200. About two-third of the non-redundant structures had sugar chains covalently bound to the proteins (glycoprotein), and remaining ones (~400) contained sugars as non-covalent ligands (~1,200 sugar residues). The non-redundant set of sugar-protein complexes was used as the input data for a sugar-binding sites prediction system on protein 3D structures, which was also developed as a part of this study. Observed frequencies of protein atoms around sugar moiety in the non-redundant set were processed into empirical scores of sugar affinity. Glucose, galactose, mannose, *N*-acetylglucosamine, fucose, and sialic acid appeared to have enough examples in the non-redundant set for construction of the empirical scores. The prediction system searches for sugar-binding sites on target protein structures by referring to the empirical scores. Performance of the system was tested on 50 complex structures (contained 127 mono-sugar binding sites) to see if the system could detect the known binding sites. As the results, the binding sites were detected as the best prediction in 42% of the cases, and 59% of them was found among the best three predictions. A prediction was considered to be correct if the predicted residue patch of a binding site had >50% overlap to the experimentally determined patch. Because the empirical scores are numerical presentation of sugar-binding site structure, they can be used to analyze (calculate) how protein recognize and discriminate different sugar types.

(59) **Histochemistry in Comparative Glycomics: Surveying the Forest Before Examining Trees**

Ajit Varki and Nissi M. Varki
Glycobiology Research and Training Center, Departments of Pathology, Medicine and Cellular & Molecular Medicine,
University of California, San Diego, La Jolla CA, USA.

The emerging science of Glycomics promises to do for Glycobiology what Genomics did for Genetics - provide a complete catalog of all glycan molecules and glycosylation sites in a given species. However, unlike the Genome, which is a linear sequence that remains essentially unchanged in a given species, the Glycome is a far more complex entity, showing marked variations in time and space. Available evidence also suggests that there are more intra- and inter-species differences. This diversity and complexity is especially prominent in the sequences of outer ends of N- and O-Glycans and Glycosphingolipids (e.g., sialylation, fucosylation, alpha-galactosylation) and in modifications of the glycans (e.g., sulfation, O-acetylation, de-N-acetylation). Furthermore, there are many cell types within each organ or tissue from a given species, each of which express a different array of glycan structures and modifications. Current approaches to Glycomics mostly involve extraction and release of total glycans of a given class from whole tissues with mass-spectrometric profiling, typically after O-methylation. These powerful methods are yielding a wealth of useful data regarding major differences between organs and between species. However, this approach does not define differences in glycan content of different cell types within the tissue studied. Also, a major and highly specific modification of a minor cell type in a tissue can be missed. Finally, the methylation procedure can destroy some glycan modifications. We suggest that current Glycomic methods be complemented by and correlated with in situ histochemical and immunoflourescent methods that use a variety of natural and recombinant glycan-recognizing probes to characterize the cell-type specific expression of each kind of glycan structure and/or modification within the tissue being examined. The specificity of such probes allows even some isomeric glycans with identical mass compositions to be distinguished. Frozen sections studied with different fixation methods can also differentially extract some glycoconjugate classes like glycosphingolipids. As mass-spectrometric techniques become more sensitive, one could envisage a day when laser-capture micro-dissection of specific cells from tissue sections might be used to marry the two approaches of in-situ localization and structural profiling. In our own collaborative work over the last decade or so, we have encountered many examples where histochemical and immunoflourescent approaches have revealed interesting features of the glycome of specific cell types that might not have been discovered using other methods. This talk will present some published and unpublished examples, with an emphasis on the biology of sialic acids in primate and rodent evolution.

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(60) **Glycan Tree Alignment and Substitution Matrix for Finding Relationships Between Glycan Linkages**

Kiyoko F. Aoki, Minoru Kanehisa and Hiroshi Mamitsuka
Institute for Chemical Research, Bioinformatics Center, Gokasho, Uji, Kyoto 611-0011 Japan.

Sequence alignment algorithms abound in bioinformatics, and from these programs, substitution matrices, or score matrices, were developed, such as PAM and BLOSUM, which helped reveal those amino acids that were deemed to be “similar” to one another. The field of glycomics is still at an early stage in its development, but we can learn from history and get closer to the current status of sequence alignment methodologies quickly. As a first step in glycome informatics, we had previously developed a glycan tree alignment procedure using dynamic programming techniques similar to the Smith-Waterman algorithm for sequence alignment. We obtained satisfactory results, but in the pursuit of more biologically meaningful alignments, we proceeded to develop a substitution matrix based on glycan alignments.

We were initially faced with many challenges due to the nature of our dataset. Firstly, the classes of glycans are not necessarily defined by structure identity like protein sequence families. Therefore, we first systematically determined the classes or “blocks” of structures to use in our procedure. Secondly, the linkage information for all structures is not completely known. Therefore, we developed a set of rules to represent our linkage information such that we did not lose too much information on the one hand, and on the other, we did not maintain such detailed information (such as possible hydroxyl group linkage information, or hypothetical versus completely unknown information, etc.) that it would become burdensome to utilize.

Once we generated our matrix in a manner similar to BLOSUM, we validated it in four ways:

1. The ability of our matrix to distinguish between different classes of glycans.
2. The distribution of alignment scores compared to that of sequence alignment scores.
3. The performance of the glycan alignments using our matrix compared to the original alignment algorithm without the matrix in terms of biological meaning.
4. An examination of the log-odds scores in reference to the literature.

These log-odds scores indicate those linkages that substituted more frequently with one another, thus signifying linkages that are similar to one another. For example, as the most prominent class of glycans, the core

linkages of N-Glycan structures scored highly by aligning with each other most often, and the fucosylated linkage on the chitobiose core scored at the top, emphasizing the rigidity of the linkages in this core structure. Other prominent linkages cited in the literature were also found. We further analyzed other aligned linkages that were different from one another.
These substitution scores are extremely useful as the basis for further understanding not only the glycan biosynthesis process, but also for characterizing glycans in a manner that could enable future research in new directions. For example, they are clues to the function of corresponding glycosyltransferases, and they may also be used to categorize the physico-chemical properties of glycans. By adjusting our glycan selection and linkage definitions in our procedure, other properties of glycans can also be characterized and examined. Consequently, the application of our methodology may certainly be considered the start of a new trend for glycomics.

(61) **Developing a Human Colonic Adenocarcinoma Cell Line with Unique Glycosylation Pattern as a Model System to Implement Concerted Functional Glycomics and Glycoproteomics Analysis**

Chi-Hung Lin[1], Chun-I Chen[1], Mei-Chun Yang[2], Hui-Chung Liang[1], Chia-Wei Lin[1], Sz-Wei Wu[1], Tong-Hsuan Chang[2] and Kay-Hooi Khoo[1]
[1] Institute of Biological Chemistry, Academia Sinica, Nankang, Taipei 11529, Taiwan, [2] GlycoNex Inc., Taiwan, Hsi-Chih City, Taipei County 221, Taiwan.

Tumor associated aberrant glycosylation often involves abnormal expression of lacto-series type 1 -(3Galβ1-3GlcNAcβ1-)$_n$ and/or type 2 -(3Galβ1-4GlcNAcβ1-)$_n$ chain, concomitant with altered degree of sialylation and fucosylation. While the type 2 poly-*N*-aectyllactosamine chain has been found in branched and/or linear extended form in both normal and cancer cells, the type 1 chain is generally considered not to occur in an extended form. Notable exceptions are the documented presence of extended type 1 chains as Le$^{b/a}$-Le^a on lactosylceramides of human colonic adenocarcinoma cell line Colo205. The possible presence of similar epitopes on extended type 1 chain of glycoproteins have been implicated by monoclonal antibody detection but no specific N- or O-glycans bearing this epitope from Colo205 or any other sources has ever been identified. In our concerted effort to develop mass spectrometry (MS) based glycomics profiling and sequencing, we first detected candidate glycans carrying such epitopes on both lactosylceramides and glycoproteins of Colo205. Subsequent detailed MS and MS/MS sequencing coupled with chromatographic fractionation, chemical derivatization and enzymatic digestion not only confirmed the presence, as major components, of mono- and dimeric Le^b/Le^a bearing lactosylceramides but also led to identification of larger lactosylceramides with up to at least six Lewis units. These multimeric Le^a were further shown to occur as both linear and branched forms. For potential therapeutic applications, humanized monoclonal antibodies were derived among which is one that specifically recognizes not single or dimeric Le^a but larger epitopes with a minimal of three Lewis units. Flow cytometric analysis demonstrated that the implicated epitopes are expressed at significantly higher level on Colo205 than several other colon caracinomas and their glycoproteins derived thereof but not on SW1116 or normal human monocytes, granulocytes, and lymphocytes. This provided a comparative basis for further glycomics and glycoproteomics analysis, for both MS methodologies development and glycobiology investigations. As first steps, we showed that dimeric Le^a-Le^a and Le$^{a/x}$-Le^x epitopes preferentially extend from the 3-and 6-arms of core 2 O-glycan structures, respectively, which echoes an early work demonstrating similar biosynthetic selectivity on the human ovarian cyst mucins. For the N-glycans, we successfully identified extended type 1 chains conjugated to internal type 2 unit susceptible to endo-β-galactosidase digestions. Using the derived monoclonal antibodies, we then implement a concerted immuno-affinity step to selectively isolate glycans, glycopeptides and glycoproteins carrying at least a trimeric Le^a structure from Colo205 and to compare its glycomics profile against other colonic carcinomas. While Le^a epitope is widely distributed among the glycoproteins extracted from Colo205, multimeric Le^a and sialyl Le^a show a more restricted expression pattern which allows a meaningful targeted glycoproteomics approach to identify both protein- and host-specific factors regulating the aberrant expression of the multimeric Lewis epitopes on unusually extended type 1 chains, and their possible immunological significance.

(62) **Sample Preparation and Mass Spectral Fragmentation Methods for Use in Glycobiology**

Catherine E Costello[1,2], Krystyn E Blackmun-Ross[1], Bogdan A Budnik[2], Yuri Elkin[1], Vera B Ivleva[1], Judith A Jebanathirajah[1], Jason L Pittman[1], Bo Xie[1], Mark E McComb[2], Peter B O'Connor[1,2] and Joseph Zaia[1]
[1] Mass Spectrometry Resource, Dept of Biochemistry, Boston Univ. School of Medicine, [2] Cardiovascular Proteomics Center, Boston Univ. School of Medicine.

New hardware and new or improved approaches to microscale sample preparation are continuously lowering the sample requirements for mass spectral structural determinations, but most of the new technology targets protein analyses. The central focus for our laboratory is the development and application of sample preparation, MS and tandem MS/MS protocols, including ion-electron fragmentation reactions, for oligosaccharides and glycoconjugates. We present here some recent results from our work that specifically address the needs of glycobiology. Release and permethylation of glycoprotein glycans: Efficient methods for sample purification and for the release and derivatization of glycoprotein glycans makes more practical the thorough characterization of glycoprotein glycans. We are optimizing multidimensional separation methods for mixtures of intact proteins/glycoproteins, and the peptides/glycopeptides and oligosaccharides that can be released enzymatically. Because permethylation increases sensitivity and helps to direct fragmentation into informative channels, we are also continuing to lower the sample requirements and minimize the formation of side products, for solution reactions and for carbohydrates released in-gel. QoTOF MS^n and qQq-FTICR MS^n: We employ combinations of mass analyzers to achieve multistage mass spectral analysis and to improve performance at each stage. We are optimizing commercial quadrupole orthogonal TOF MS instruments, with both MALDI and ESI sources, for glycobiology and are also making use of a novel qQq-FTICR MS constructed in-house. With this hybrid instrument, ions can be isolated in the mass resolving Q1 and accumulated in Q2 and fragmented there or transmitted to the FT cell for fragmentation and mass analysis. Minor components can be accumulated in Q2, allowing their further analysis with high sensitivity and without interference from more abundant components. Both low-energy CID in Q2 and the full range of FT fragmentation methods (see below) are available for unambiguous structural determinations of the trapped ions. High pressure, vibrationally cooled (VC) MALDI-FTMS: VC MALDI-FTMS generates ions by desorbing them with simultaneous gas pulses that bring the pressure at the target to the ~1-10 mBar range. This method allows detection of labile species with FTMS resolution and accuracy, and facilitates studies of complex biological molecules, particularly when labile substituents are present, as glycan substituents or as post-translational modifications to proteins and peptides. VC-MALDI also demonstrates improved production of multiply charged ions, which are generally advantageous for MS/MS experiments. Fragmentation methods: Structural determinations of both ESI-generated molecular ions and singly and multiply charged HP MALDI ions can be facilitated by using ion-electron reactions and tandem mass spectrometry of even electron ions with labile groups. Electron-based fragmentation can be used alone or in conjunction with collision-induced decomposition (CID) and/or photodissociation (PID). It is important to note that the electron-based methods often leave intact fragile bonds that cleave under other conditions, so that labile groups are still present in the fragments. Applications: We are developing these methods with well-characterized glycans and glycoconjugates and are applying them to the structural determinations of unknown samples from biological sources. Included in ongoing studies are glycolipids, Lipids A, *N*- and *O*-linked glycoproteins. Examples from these classes will be shown.

(63) **Development of Structural Analyses of Sulfated *N*-glycans by Mass Spectrometry and HPLC Mapping**

Hirokazu Yagi[1], Noriko Takahashi[1], Yoshiki Yamaguchi[1], Naoko Kimura[2], Reiji Kannagi[2] and Koichi Kato[1]
[1] Graduate School of Pharmaceutical Sciences, Nagoya City University, Japan, [2] Division of Molecular Pathology, Aichi Cancer Center, Japan.

Multi-dimensional HPLC mapping method using pyridylaminated (PA) *N*-glycans which we have been developing is a forceful method to identify the structures of *N*-glycans released from proteins based on their elution positions on the three different HPLC columns. HPLC data of approximately 500 different *N*-glycans accumulated so far are now available in a web application, GALAXY (http://www.glycoanalysis.info/). However, it is still difficult to identify the structures of sulfated oligosaccharides, which play a crucial role in cell-cell interactions, because little or no HPLC data of these oligosaccharides are obtained as yet. Here

we aimed at collecting the structural data of sulfated *N*-glycans to extend applicability of the HPLC map.
Since it was difficult to prepare sulfated oligosaccharides from natural sources due to their low abundance, we utilized a bladder carcinoma cell line (LS12), which were cotransfected with both GlcNAc 6-*O*-sulfotransferase-1 (GlcNAc6ST-1) and α 1-3 fucosyltransferase VII cDNAs. The newly produced *N*-glycans were released from the membrane protein of LS12 by glycoamidase A digestion. The released glycans were labeled with 2-aminopyridine and then digested with sialidase. The digestion products were applied onto an anion-exchange column to fractionate anionic asialooligosaccharides, which were further separated by ODS and amide-silica columns. The isolated anionic glycans were subjected to mass spectrometric (MS) analysis, glycosidase digestions, and desulfation by HCl treatments for structural analyses. The MS data indicated that all anionic asialooligosaccharides derived from LS12 were sulfated. We elucidated 18 different sulfated oligosaccharide structures, which are bi-, tri-, and tetraantenary complex-type glycans with the GlcNAc residue(s) sulfated at the C6 position. Furthermore, we identified 8 sulfated oligosaccharides generated by various glycosidase treatments of the original sulfated oligosaccharides. All monosulfated glycans derived from LS12 had a sulfate group on the GlcNAc residue in the GlcNacβ 1-2Manα 1-3 branch, while the disulfated oligosaccharides had sulfate groups on the GlcNAc residues in the GlcNacβ 1-2Manα 1-3 and GlcNacβ 1-2Manα 1-6 branches. These results indicate that GlcNAc6ST-1 transfers the sulfate group preferentially to the GlcNAc residue in the GlcNacβ 1-2Manα 1-3 branch. Hence, the HPLC data of more than 25 kinds of sulfated oligosaccharides are now available, which facilitates structural analyses of *N*-glycans including sulfated oligosaccharides.
This work was supported by CREST/JST.

(64) **Printed Covalent Glycan Array for Ligand Profiling of Diverse Glycan Binding Proteins**

Ola Blixt[1], Steve Head[2], Tony Mondala[2], Christopher Scanlan[2], Richard Alvarez[3], Marian C. Bryan[2], Fabio Fazio[2], Daniel Calarese[2], James Stevens[2], Nahid Razi[1], Irma van Die[4], Dennis Burton[2], Ian A, Wilson[2], Richard Cummings[3], Margaret E. Huflejt[5], Nicolai Bovin[6], Chi-Huey Wong[2] and James C. Paulson[2]
[1] Glycan Synthesis and Protein Expression Core D, Consortium for Functional Glycomics, Department of Molecular Biology, CB216, The Scripps Research Institute, 10550 North Torrey Pines Road, 92037, La Jolla, USA, [2] The Scripps Research Institute, [3] University of Oklahoma Health Science Cntr., Biochemistry & Molecular Biology, Oklahoma City, [4] Department of Molecular Cell Biology & Immunology, VU medical center, Amsterdam, the Netherlands, [5] Sidney Kimmel Cancer Center, San Diego, [6] Shemyakin & Ovchinnikov Institute, Russian Academy of Sciences, Moscow.

Glycan binding proteins (GBP) play a significant role in recognizing and specifically binding to glycosylated protein and lipid ligands. To understand the nature of GBP-ligand interactions that underlie the biological roles of these proteins several approaches have been taken for construction of glycan arrays for analysis of GBP specificity (1,2). Each format differs in the manner in which the glycans are displayed, and in the type of glycans displayed. The Consortium for Functional Glycomics (CFG) has developed a novel glycan array format that employs covalent coupling of glycans to glass slides. The array utilizes standard robotic printing technology and commercial amine reactive N-hydroxy-succinimido (NHS) activated glass slides which allows rapid covalent coupling of amine functionalized glycans or glycoconjugates. To construct a diverse array, advantage has been taken of the availability of a library of synthetic (3) and natural structurally defined terminal sequences of glycoprotein and glycolipid glycans available from the CFG. This diverse glycan array has been demonstrated to have applicability for profiling the specificity of a wide variety of GBPs including mammalian lectins (C-type lectins, galectins and siglecs), plant lectins, antibodies and viral and bacterial lectins. Application of GBPs to the array typically requires amplification of valency to stabilize binding for intrinsic affinities of the GBPs for their prerfered ligands (Kd= 1-1000 μM), mimicking the biologically relevant multivalent interactions that occur in nature. The array requires small amounts of GBP for analysis (5-10 μg) and can be readily expanded to more than 1000 glycans as they become available. Analysis on this array format will be available through the Consortium for Functional Glycomics (http://www.functionalglycomics.org). [1] Drickamer, K. and Taylor, M.E. (2002) Glycan arrays for functional glycomics. Genomebiology, 3, 1034.1031-1034. [2] Feizi, T., Fazio, F., et al. (2003) Carbohydrate microarrays-a new set of technologies at the frontiers of glycomics. Current Opinion in Structural Biology, 13, 637-645. [3] Blixt, O. and Razi, N. (2004) Strategies for Synthesis of an Oligosaccharide Library Using a Chemoenzymatic Approach. In Wang, P.G. and Ichikawa, Y. (eds), Synthesis of Carbohydrates through Biotechnology. American Chemical Society,Washington DC, pp. 93-112.

(65) **Glycan Profiling by Means of Lectins**

Jun Hirabayashi.
Research Center for Glycoscience, AIST Tsukuba Central 6, 1-1-1, Higashi, Tsukuba, Ibaraki 305-8566, Japan.

Glycans are the third bioinformative macromolecules, of which structures are constructed by a completely different principle from that for nucleic acids and proteins, i.e., by a multi-genes/multi-glycan rule. Moreover, glycans are not linear molecules like the others, and the former molecules have various isomeric linkages including anomerism, which the latter ones lack. Therefore, there should be a distinct approach to characterize glycan structures, which meets the idea of "glycan profiling". Mass spectrometry can be a powerful method to study complex glycans if distinct fragmentation patterns of individual glycans are reproducibly analyzed. However, its application to sialylated glycans and glycoconjugates, scuh as glycopeptides and glycoproteins still has practical difficulty. A promising alternative is the use of carbohydrate-recognition proteins (i.e., lectins) from a more quantitative viewpoint, because lectins are known to exert relatively a wider range of affinities to a series of glycans having related structures, when compared with antigen-antibody interactions. However, this never means that such "loose specificity" is not useful as tools for glycan profiling, if they reproducibly show significantly different affinities to different saccharides. Moreover, since lectins are endogenous molecules playing critical roles in vivo, basic studies of lectins should also contribute to understanding their physiological roles. "Hect-by-hect" project has been in progress to define 100 lectins vs. 100 glycans interactions in terms of dissociation constant (Kd) by using reinforced frontal affinity chromatography. The results obtained so far clearly demonstrate that the concept of "glycan profiling" by lectins proved to be true from a practical viewpoint, too. This work is undertaken along with the SG (structural glycomics) project, in the framework of NEDO (New Energy and Industrial Technology Organization) under the METI (the Ministry of Economy, Trade, and Industry), Japan.

(66) **Synthesis and Utilization of Trisaccharide Library in Sequencing of Oligosaccharide.**

Osamu Kanie.
Mitsubishi Kagaku Institute of Life Sciences (MITILS) Machida-shi, Tokyo 194-8511 Japan.

Sequencing methodology based on mass spectrometry for the oligosccharide is of extreme importance. Especially one capable of elucidating configurations and positional isomers, of which information cannot be obtained by ordinary methods, is needed for the structural analysis of newly discovered natural products as well as to match current trend of proteomics research with posttranslational modifications. Further, carbohydrates can be considered as an important class of potent pharmaceuticals since wide range of functions involved in cellular interactions is being discovered. Despite diverse functions, only few examples have been focused as candidates of oligosaccharide based pharmaceuticals. Recent efforts directed toward the synthesis of complex glycoconjugates made it possible to control stereo- and regio-specificity in the glycosylation reactions using solution phase and solid phase organic synthesis. The state-of-the-art synthesis of oligosaccharide, however, cannot directly be applied to a combinatorial chemistry in conjunction with high throughput screening to discover pharmaceutical leads.

In our efforts to address these issues, we have initiated a study to understand structural information contained in the oligosaccharide. Our approach is based on analysis of synthetic oligosaccharides using mass spectrometer at MS^n. Topics in the synthetic approach towards tisaccharide library and the use in an approach to elucidate anomeric configurations based on mass spectrometry will be discussed.
SYNTHESIS: We have reasoned one of the difficulties of oligosaccharide library synthesis to be the use of a variety of protecting groups in a monosaccharide unit, which sometimes causes pealing of protecting groups. Thus, we decided to use benzyl group as a sole protecting group for required units except for anomeric position. Furthermore, such units were

armed with phenylthio group or fluorine at anomeric center, which facilitate the orthogonal glycosylation system. We have synthesized complete library of tirsaccharides consisting of all combination of anomers based on solid phase synthesis under newly introduced diffusive reaction conditions.
ANALYSIS: The process of collision induced dissociation mass spectrometer is radical dissociation reaction. The reaction must be governed by bond energy. In order to experimentally confirm this, we have used our synthetic library compounds as a source of structurally defined trisaccharides, and carried out MS^n analyses using quadrupole ion trap mass spectrometer where amplitude of RF voltage was varied. As a result, it was shown that the dissociation curves thus obtained reflect the anomeric configurations.
This research was supported by Key Techology Research Promotion Program, The New Energy and Industrial Development Organization (NEDO), Ministry of Economy, Trade and Industry (METI) of Japan.

(67) **A Subtype of Human Gastric Mucins Expressing alpha1,4-GlcNAc-capped *O*-glycans Functions as a Natural Antibiotic Against *Helicobacter pylori* Infection**

Jun Nakayama[1], Masatomo Kawakubo[1], Yuki Ito[1], Motohiro Kobayashi[1,3], Michiko N Fukuda[3], Minoru Fukuda[3] and Tsutomu Katsuyama[2]
[1] Department of Pathology, Shinshu University School of Medicine, [2] Department of Laboratory Medicine, Shinshu University School of Medicine, [3] Glycobiology Program, Cancer Research Center, The Burnham Institute.

Helicobacter pylori (*H. pylori*) largely colonizes the surface mucous cell-derived mucin while this bacteria are barely found in gland mucous cell-derived mucin secreted from the deeper portions of the gastric mucosa (1). Because the gland mucous cells characteristically produce terminal alpha1,4-GlcNAc residues on core2-branched *O*-glycans (2), we have investigated the role of these unique *O*-glycans on *H. pylori* infection (3). Thus, recombinant soluble CD43 (sCD43) having terminal alpha1,4-GlcNAc was prepared by transfecting CHO cells with alpha1,4-*N*-acetylglucosaminyltransferase (alpha4GnT), core2 beta1,6-*N*-acetylglucosaminyltransferase, and soluble CD43 cDNAs. Similarly, control sCD43 was prepared using pcDNAI instead of alpha4GnT cDNA for transfection. When *H. pylori* was incubated with the alpha1,4-GlcNAc-capped sCD43, the growth and motility of the bacteria was significantly suppressed, and the abnormal morphology such as elongation and folding were found. Such inhibitory effects were also observed when the natural mucins having alpha1,4-GlcNAc-capped *O*-glycans prepared from human gastric mucosa were tested, whereas the natural mucin derived from the surface mucous cells rather stimulated the growth of *H. pylori*. By contrast, the control sCD43 had no effects on bacterial growth and morphology (3). The cell wall of *H. pylori* characteristically contains a unique glycolipid, cholesteryl-alpha-D-glucopyranoside (CGL) (4). We found that alpha1,4-GlcNAc-capped *O*-glycans suppressed the synthesis of CGL by inhibiting the activity of UDP-Glc:sterol alpha-glucosyltransferase responsible for the biosynthesis of CGL *in vitro*. In *H. pylori*, cholesterol is utilized for the biosynthesis of CGL, whereas genes involved in the *de novo* synthesis of cholesterol are not found in the bacteria. When cultured in the absence of cholesterol, *H. pylori* lacked CGL, exhibited very reduced growth and motility, and died off completely upon prolonged incubation up to 21 days. These results established that exogenous cholesterol is required for the biosynthesis of CGL and survival of *H. pylori*. In order to test whether alpha1,4-GlcNAc-capped *O*-glycans protect host cells from *H. pylori*, gastric adenocarcinoma AGS-alpha4GnT cells stably expressing alpha1,4-GlcNAc-capped *O*-glycans as well as mock-transfected AGS cells were co-cultured with *H. pylori*. We found that alpha1,4-GlcNAc-capped *O*-glycans do not modify the adhesive property of *H. pylori* to AGS-alpha4GnT cells. However, AGS-alpha4GnT cells seemed to be intact, and *H. pylori* barely grew. By contrast, the marked cellular damage was observed in mock-transfected AGS cells with surrounding *H. pylori*. These results combined together indicate that alpha1,4-GlcNAc-capped *O*-glycans secreted from the gastric gland mucous cells apparently function as a natural antibiotic against *H. pylori* by inhibiting the biosynthesis of CGL, thus protecting the gastric mucosa from the infection by *H. pylori* (3). Supported by a Grant-in-Aid for Scientific Research on Priority Area 14082201 from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by grants CA 71932 and CA 33000 from the National Cancer Institute. (1) Hidaka et al, *Gut* 49, 474-480, 2001 (2) Nakayama et al, *Proc Natl Acad Sci USA* 96, 8991-8996, 1999 (3) Kawakubo et al, *Science* 305, 1003-1006 (4) Hirai et al, *J Bacteriol* 177, 5327-5333, 1995

(68) **Carbohydrate Microarrays as Versatile Tools for Glycobiology**

Peter H. Seeberger and Matthew D. Disney
ETH Z¸rich, Laboratory for ORganic Chemistry, HCI F315, Wolfgang-Pauli-Str. 10, 8093 Zurich, Switzerland .

The growing field of glycomics is suffering from the lack of molecular tools for screening, imaging, purification and other procedures that are routine in studies involving peptides and oligonucleotides. Using an automated oligosaccharide synthesizer we developed some time ago, access to defined oligosaccharides has become very rapid. These synthetic molecules, as well as any isolated carbohydrate, can now be readily converted into a series of tools that aid biological and medical investigations. Described are microarrrays and their use to 1) Detect pathogenic bacteria; 2) Determine lectin fingerprints; 3) Screen RNA-carbohydrate interactions in the search for better antibiotics. In addition, carbohydrate-containing fluorescent polymers were used to detect low levels of bacteria in body fluids such as blood. Disney, M.D.; Seeberger, P.H.; Carbohydrate Arrays to Determine Cell-Pathogen Adhe-sion Profiles and To Detect Pathogens; submitted for publication. Disney, M. D.; Seeberger, P. H.; Aminoglycoside Microarrays to Explore Carbohydrate-RNA Interactions, Chem. Eur. J. 2004, 10, 3308-3314. Disney, M.D.; Magnet, S.; Blanchard, J.S.; Seeberger, P.H. Aminoglycoside Microarrays to Study Antibiotic Resistance, Angew. Chem. Int. Ed. 2004, 43, 1591-1594. Disney, M.D.; Zheng, J.; Swager, T.; Seeberger, P.H.; Visual Detection of Bacteria with Carbohydrate Containing Fluorescent Polymers, J. Am. Chem. Soc. 2004, 126, in press.

(69) **Profiling Glycosylation using Azidosugars in Vivo**

Danielle H Dube, Jennifer A Prescher, Chi N Quang and Carolyn R Bertozzi
Department of Chemistry, University of California, Berkeley, CA 94720.

Metabolic oligosaccharide engineering employs the cell's biosynthetic machinery to install unnatural monosaccharides into cellular glycans (1, 2). Briefly, synthetically-derived unnatural monosaccharides intercept permissive carbohydrate biosynthetic pathways, are transformed into activated sugars, and ultimately are appended to glycans destined for cell surface presentation. Using this technique, our lab has demonstrated that monosaccharides containing the bioorthogonal azide group can be metabolically introduced into cellular glycans through several different carbohydrate biosynthetic pathways. For example, the mannosamine-based substrate peracetylated N-azidoacetylmannosamine (Ac_4ManNAz) transits through the sialic acid biosynthetic pathway and is converted to N-azidoacetyl sialic acid (SiaNAz), leading to the display of azides in sialic acid residues (3). In contrast, peracetylated N-azidoacetylgalactosamine (Ac_4GalNAz) is incorporated into the core position of mucin-type O-linked glycoproteins (4), and peracetylated N-azidoacetylglucosamine (Ac_4GlcNAz) is installed onto proteins containing the O-GlcNAc modification (5). In each of these cases, the azide can undergo Staudinger ligation with exogenously delivered phosphine reagents for the attachment of biological probes (3). Azidosugars and the Staudinger ligation have been used in cell-based systems to perturb biological interactions, target modified cell surfaces, probe metabolic flux, and identify specific glycoprotein subtypes from the proteome (1). Most recently, we have demonstrated that Ac_4ManNAz is metabolically converted to SiaNAz in vivo, and the product SiaNAz can be chemically detected in live mice (6). We have further investigated the metabolism of Ac_4GalNAz, Ac_4GlcNAz, and SiaNAz in vivo, and the results are reported here. We have found that Ac_4GalNAz and SiaNAz are metabolically incorporated onto splenocyte cell surfaces and into tissue glycoproteins, but Ac_4GlcNAz is not incorporated into glycans at detectable levels. Ac_4GalNAz and SiaNAz label distinct glycans as determined by western blot, consistent with the notion that these sugars have different metabolic fates. Interestingly, unlike Ac_4ManNAz, Ac_4GalNAz and SiaNAz are metabolized more efficiently in B-cells versus other splenocyte subpopulations. We are currently investigating whether this distinct labeling is based on differences in metabolic turnover, glycoprotein hosts and/or access to the unnatural sugars. Additionally, efforts are underway to identify the azide-labeled glycoprotein species (both the glycan and underlying protein) and to tag them in live mice using Staudinger ligation for all azidosugar inputs. The ability to tag cell surface glycans in vivo may enable therapeutic targeting and noninvasive imaging of changes in glycosylation during disease progression. References: (1) D. H. Dube, C. R. Bertozzi, Curr. Opin. Chem. Biol. 7, 616-625 (2003). (2) O. T. Keppler, R. Horstkorte, M. Pawlita, C. Schmidts, W. Reutter, Glycobiology 11, 11R-18R (2001). (3) E. Saxon, C. R. Bertozzi, Science 287, 2007-2010 (2000). (4) H. C. Hang, C. Yu, D. L. Kato, C. R. Bertozzi, Proc. Natl. Acad. Sci. U.S.A. 100, 14846-14851 (2003). (5) D. J. Vocadlo,

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(70) **Carbohydrate-based Cancer Vaccines**

Yanbin Pan[1], Peter Chefalo[2], Nancy Nagy[2], Clifford Harding[2] and Zhongwu Guo[1]

[1] Department of Chemistry, Case Western Reserve University, 10900 Cleveland, OH 44106, USA, [2] Department of Pathology, Case Western Reserve University, 10900 Cleveland, OH 44106, USA.

Tumor cells express special carbohydrates, which are defined as tumor-associated carbohydrate antigens (TACAs). TACAs are important molecular targets for the development of cancer vaccines or cancer immunotherapies, which are considered ideal treatments for tumors, owing to their high efficiency and specificity. Several carbohydrate-based cancer vaccines are now on clinical trials. However, among numerous TACAs identified so far only a few can be used for cancer vaccine development. An important problem with the majority of TACAs is immunotolerance, i.e., TACAs as well as their conjugates fail to induce immune responses in cancer patients. This problem has severely hindered further progress in the study of cancer vaccines and cancer immunology. To overcome this problem, we recently developed a new strategy that is based upon glycoengineered modification of cancer cells. First, an artificial derivative (neoantigen) of a TACA is prepared and used to vaccinate cancer patients. Once an immune response specific to the neoantigen is established, the patients are treated with the correspondingly modified monosaccharide precursor of the target TACA to initiate the exclusive expression of the neoantigen on tumor cells. The provoked immune system will thus react to eradicate the specifically marked tumors. Immunologic studies of chemically modified GM3 antigens indicated that the artificial TACAs are much more immunogenic than the natural counterparts, while systemic studies of N-acyl mannosamines showed that several derivatives are good precursors for the glycoengineering of cancer. Moreover, in vitro and in vivo studies using RMA and other leukemia tumor models with α(2,8)polysialic acid as the target TACA and N-propionyl mannosamine as the bioengineering precursor proved that: 1) chemically modified TACAs can form very effective cancer vaccines; 2) tumors and TACAs on tumor cells can be efficiently glycoengineered both in vitro and in vivo with chemically modified monosaccharide as precursors; 3) the immune responses and cytotoxicity induced by the synthetic vaccines are specific to glycoengineered tumors; 4) the new immunotherapy (treating tumor animals with antibodies and modified monosaccharide precursors) can suppress tumor growth and completely inhibit tumor metastasis.

(71) **Oligosaccharide-MTX Conjugate for the Analyses of UGGT Mediated Glucosylation**

Kiichiro Totani[1], Yoshito Ihara[2], Ichiro Matsuo[1,2] and Yukishige Ito[1,2]

[1] RIKEN (The Institute of Physical and Chemical Research), Wako, Saitama 351-0198 Japan Saitama 351-0198, Japan , [2] Nagasaki University School of Medicine, Nagasaki 852-8523 Japan, [3] CREST (JST), Kawaguchi 322-1102 Japan.

Oligosaccharide parts of glycoproteins play important roles in a variety of biological events. In particular, the functions of high-mannose type oligosaccharides in ER glycoprotein quality control are attracting recent attention [1]. In order to understand their functions precisely, glycoprotein-related molecular probes having homogeneous and structurally defined oligosaccharide are desired. We recently developed a novel type of the artificial glycoprotein that consists of oligosaccharide-MTX hybrid molecule and dihydrofolate reductase (DHFR). MTX has been known as a strong inhibitor of DHFR and the tight binding affinity can be applied to create various oligosaccharide-grafted DHFR [2]. We now wish to report the use of MTX-bound oligosaccharide as a substrate of UDP-glucose: glycoprotein glucosyltransferase (UGGT), which is one of the key components in glycoprotein quality control. The endoplasmic reticulum (ER) is the entrance of newly synthesized proteins to secretory pathways. ER lumen is the post-translational modification machinery having intricate quality control system, which assists correct folding of proteins and prevents the aggregation and the transport of misfolded glycoproteins to the Golgi complex. So-called calnexin (CNX)/calreticulin (CRT) cycle plays the central role in glycoprotein quality control. CNX is a membrane-bound molecular chaperone and CRT is its soluble homologue. Both CNX and CRT have a lectin property and recognize monoglucosylated high-mannose type glycan Glc1Man9GlcNAc2 (G1M9) of asparagine (Asn)-linked glycoproteins as a primary ligand. In CNX/CRT cycle, UDP-glucose: glycoprotein glucosyltransferase (UGGT) [3] plays a pivotal role as the folding sensor. It specifically glucosylates Man9GlcNAc2-N-glycans (M9) of incompletely folded glycoprotein to regenerate CNX/CRT ligand, Glc1Man9GlcNAc2 (G1M9). The major difficulty in the analyses of UGGT has been the lack of well-defined substrate that can be accepted by UGGT. We found that M9-MTX and its DHFR conjugate can be accepted as substrates of UGGT-mediated glucosylation. With this substrate, full characterization of the UGGT product was realized for the first time, by using HPLC, MALDI-TOF-MS, and 1H-NMR. [1] Ellgaard, L. & Helenius, A. Nat. Rev. Mol. Cell. Biol. 4, 181-191 (2003). [2] Totani, K., Matsuo, I., & Ito, Y. Bioorg. Med. Chem. Lett. 14, 2285-2289 (2004). [3] Caramelo, J. L., Castro, O. A., Alonso, L. G., de Prat-Gay, G. & Parodi, A. J. Proc. Natl Acad. Sci. 100, 86-91 (2003); Taylor, S., Thibault, P., Tessier, D. C., Bergeron, J. M. & Thomas, D. Y. EMBO Rep. 4, 405-411 (2003); Ritter, C. & Helenius, A. Nat. Struct. Biol. 7, 278-280 (2000).

(72) **Characterization of Cargo Receptors ERGIC-53, VIP36, and VIPL using Monoclonal Antibodies**

Norihito Kawasaki, Naoki Matsumoto and Kazuo Yamamoto

Dept. of Integrated Bioscience, Grad. School of Frontier Sciences, Univ. of Tokyo.

Newly synthesized glycoproteins destined for secretion are transported from the ER through the Golgi toward the cell surface via transport vesicles. In this secretory pathway, several L-type lectins serve as cargo receptors. ERGIC-53, VIP36, and VIPL have an L-type lectin domain in their luminal part and are supposed to be such receptors. To understand function of these lectins, we prepared several monoclonal antibodies against human ERGIC-53 and VIPL. The luminal parts of human ERGIC-53, VIP36, and VIPL were transiently expressed on the surface of 293T cells, and then these cells were immunized into rat lymph nodes. The hybridoma cells producing anti-ERGIC-53 and anti-VIPL antibodies were screened and several kinds of clones were established successfully. Using the cells expressing ERGIC-53, VIP36, or VIPL on the surfaces, respectively, it was shown that the anti-ERGIC-53 monoclonal antibodies did not cross-react with VIP36 and VIPL. Anti-VIPL antibodies also demonstrated to be specific for VIPL. Recombinant MCFD2, which is the ER resident protein, specifically bound to ERGIC-53-expressing cells in the presence of calcium ions. We investigate the molecular mechanism of the interaction between ERGIC-53 and its cargo in the presence or absence of MCFD2. By using these antibodies, we also report the localization patterns of ERGIC-53, VIP36, and VIPL in the cells.

(73) **Engineering of a Thioligase: Randomized Mutagenesis of the Acid/Base Residue Leads to the Identification of Improved Catalysts.**

Johannes M Ilegger[1], Michael Jahn[1], Hong-Ming Chen[1], R. Antony J. Warren[2] and Stephen G. Withers[1]

[1] Protein Engineering Center of Excellence, Department of Chemistry, University of British Columbia, Vancouver, B. C. V6T 1Z1, Canada , [2] Protein Engineering Center of Excellence, Department of Microbiology, University of British Columbia, Vancouver, B. C. V6T 1Z1, Canada .

Enzymatic synthesis of oligosaccharides has proven to be very efficient compared to classical chemical synthesis. Recently thioligases have been added to the useful repertoire of such enzymatic tools ((2003) Angew. Chemie, 42, 352-354). These catalysts are retaining glycosidases in which the acid/base residue has been mutated. The enzyme is still capable of forming a covalent enzyme-glycosyl intermediate when a substrate with a good leaving group such as dinitrophenol is provided. Subsequent transfer of the glycosyl moiety onto a highly nucleophilic thiosugar acceptor forms a thiolinkage between the carbohydrate units. The acid/base mutant E170A of *Agrobacterium* sp. fl-glucosidase (Abg) was shown to be a very effective thioligase. Here we report the randomized mutagenesis of the acid/base catalyst E170 and identification of the variant E170Q that is a superior thioligase, with reactions that are 5-fold faster when dinitrophenyl glucoside is used and 100-fold faster when glucosyl azide is used. Furthermore, different acceptor and donor sugar combinations were employed to produce thio-linked di- or trisaccharides in high yields, showing the high versatility of the system for the synthesis of carbohydrate mimetics.

(74) **Solid-Phase Synthesis of the Glycopeptide Bearing Consecutive N- and O-glycans**

Yuko Nakahara, Hironobu Hojo and Yoshiaki Nakahara

Department of Applied Biochemistry, Institute of Glycotechnology, Tokai University.

With the advent of high performance instruments, there has been a growing demand for application of the mass spectral measurement in the analysis of the intact glycosyl surface of functional glycoprotein. Synthetic glycopeptide of unambiguous structure serves as an indispensable tool for establishing the analytical method to gain the reliable data. We report here synthesis of a glycopeptide fragment bearing consecutive N- and O-glycans that represents highly glycosylated domain of human glycophorin.

(75) **A Practical Synthetic Approach Towards Core 2 O-glycan-linked Glycopeptides**

Yutaka Takano, Hironobu Hojo, Naoya Kojima and Yoshiaki Nakahara
Department of Applied Biochemistry, Institute of Glycotechnology, Tokai University, Kitakaname 1117, Hiratsuka, Kanagawa, 259-1292, Japan .

Due to the biological significance and the challenging structural features, we have studied synthesis of the core 2 O-glycan-linked glycopeptides. Here, we demonstrate our strategy comprised of benzyl protection-based synthesis of the core 2 saccharide-Ser/Thr building blocks, solid-phase synthesis of the glycopeptides, debenzylation in the specific conditions with low-acidity TfOH, enzymatic glycosylation, and peptide ligation. The intermediate, LacNTCA glycosyl donor, was extensively utilized for preparation of the LacNAc-repeating core 2 glycan.

(76) **Synthesis of Bisubstrate Type Inhibitor of *N*-Acetylglucosaminyltransferases Using Polymer-Resin Hybrid Strategy**

Shinya Hanashima[1], Shino Manabe[1,3], Kei-ichiro Inamori[2], Naoyuki Taniguchi[2] and Yukishige Ito[1,4]
[1] RIKEN (The Institute of Physical and Chemical Research), [2] Department of Biochemistry, Osaka University Medical School, [3] PRESTO, JST (Japan Science and Technology Agency), [4] CREST, JST.

Synthesis of bisubstrate type *N*-acetylglucosaminyltransferases (GnTs) inhibitor was achived.[1] GnTs are key enzymes to produce highly branched complex-type *N*-glycan structures. GnT-V transfers an *N*-acetylglucosamine (GlcNAc) residue to the core α1,6-mannose (Man) arm to form a β1,6-linkage. It is well known that the levels of β1,6-branched glycans are increased in tumor cells, and GnT-V is involved in cancer metastasis.[2] Also, relationships with T-cell activation and angiogenesis have been revealed. It is indicated that inhibitors of GnT-V may be useful in the treatment of several kinds of cancer cell lines in vitro and in vivo. In addition to gene knockout strategy, chemical inhibition would be promising approach.

Recently identified GnT-IX is a homologue of GnT-V, which is exclusively expressed in brain, and its roles in neuronal development and functions are suggested. It has a broader specificity and transfers GlcNAc to both α1,6- and α1,3-mannose as well as to *O*-linked GlcNAcβ1,2Man.[3] To elucidate the reaction mechanism and active site structure of GnT-V and IX, we designed bisubstrate type inhibitor which contains both donor (UDP-GlcNAc) and acceptor components. As the acceptor component, the trisaccharide (βGlcNAc1→2αMan1→6βMan) was incorporated, because it was previously reported by Hindsgaul et al. to serve as an efficient acceptor of GnT-V.[4] UDP-GlcNAc donor component and acceptor trisaccharide component were tethered with various length of linkers for seeking the distance between each binding sites. We intended that these designed inhibitors would be superior to the preexisting one in the respect of inhibitory activity and selectivity, because of the tightly binding into both binding pockets of GnTs.

As a primary goal of this work, a prototypical compound having sulfide tether was designed. [1] In order to approach the target, convergent route was adopted. Namely, trisaccharide and donor components were constructed separately and combined together by chemoselective ligation. It utilized polymer-resin hybrid capture-release strategy for the construction of the acceptor component.[5,6] One pot reduction, ligation, and deprotection procedure in aqueous media were used to produce the coupling product selectively from thiol induced trisaccharide component and bromoacetamide having GlcNAc component, followed construction of diphosphate linkage using UMP-morpholidate led to the target compound. Inhibitory activities toward GnT-V and IX were evaluated to reveal its potency toward the latter enzyme (K_i = 7.2 μM).

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(77) **Snapshots of the Catalytic Cycle of β-1,4-galactosyltransferase-I**

Boopathy Ramakrishnan[1,2], Velavan Ramasamy[1] and Pradman K. Qasba[1]
[1] Structural Glycobiology Section, LECB, CCR, NCI-Frederick, Frederick, MD, 21702, USA, [2] BRP, SAIC-Frederic, Inc.

β-1,4-galactosyltransferase-1 (β4Gal-T1), in the presence of Mn^{2+} transfers galactose from the sugar donor UDP-α-D-galactose (UDP-Gal) to an acceptor GlcNAc. Earlier studies have shown that during the catalytic cycle of the enzyme a flexible loop changes the structure from an open to a closed conformation. These conformational changes are initiated by the binding of Mn^{2+}, followed by UDP-Gal to the enzyme. Although the metal ion binds to the enzyme in the open conformation, its coordination to the enzyme is known only in the closed conformation with the bound UDP-Gal. We report here the crystal structure analysis of Met344His mutant of human β4Gal-T1, M344H-Gal-T1, in the open conformation as an apo-enzyme, and its Mn^{2+} bound and Mn^{2+}-UDP-Gal bound complexes. These crystal structures represent snapshots of the Mn^{2+} binding to the enzyme in an open conformation, organization of the water molecules and freezing the hinge region residues of the flexible loop, followed by UDP-Gal binding and closed conformation of the flexible loop. Furthermore, in order to capture the transition state complex in the crystal structure, we have exploited the very poor N-acetylgalactosaminyltransferase activity (GalNAc-T) of the β4Gal-T1 in the presence of α-Lactalbumin (α-LA) towards Glc. We have determined the crystal structure of β-4Gal-T1- α-LA complex in the presence of UDP-GalNAc, Mn^{2+} and Glc. In the crystal structure both the donor and acceptor substrates are clearly observed. Although the GalNAc moiety of the donor is clearly observed, it is hydrolyzed from the UDP-GalNAc and found 3.1 □ away from the P(β-) phosphate oxygen atom, displaced towards the acceptor, Glc, molecule. In the crystal structure the anomeric C1 atom of the GalNAc moiety has only two covalent bonds with the non-hydrogen atoms (O5 and C2 atoms). Therefore it seems that the GalNAc moiety exists in an oxocarbenium-like transition state. Due to the steric hindrance caused by the Try286, the hydrolyzed GalNAc moiety in its oxocarbenium-like state is placed in such a way that it is 2.8 □ away from the O4 oxygen atom of the Glc molecule, yet to form the product, β-GalNAc1-4Glc. This represents a snapshot of the catalytic transition state complex of β-4Gal-T1. More details on these crystal structures and their implications will be presented. Funded under Contract No. NO1-CO-12400.

(78) **Chain Polymerization of Heparan Sulfate on a GlcNAc-Containing Linkage Region Analog as Acceptor**

Sun-Young Park[1], Hiroshi Kitagawa[1], Jun-ichi Tamura[2] and Kazuyuki Sugahara[1]
[1] Department of Biochemistry, Kobe Pharmaceutical University, Higashinada-ku, Kobe 658-8558, Japan,
[2] Department of Regional Environment, Faculty of Regional Sciences, Tottori University, Japan.

HS co-polymerases, encoded by *EXT1* and *EXT2*, synthesize heparan sulfate chains by alternate transfer of α-GlcNAc and β-GlcA to the linkage region tetrasaccharide of proteoglycans. However, no polymerization can be achieved with a mixture of individually expressed EXT1 and EXT2 proteins as an enzyme source. However, in view of the recent findings that the cells deficient in *EXT1* could form shorter HS chains, we speculate that even EXT1 or EXT2 alone might be able to polymerize HS chains to some extent, provided that the first GlcNAc has been transferred to the linkage region. In fact, here we showed marked HS polymerization achieved by individually expressed EXT1 or EXT2 on the synthetic analog GlcNAcα1-4GlcUAβ1-3Galβ1-*O*-C_2H_4NH-benzyloxycarbonyl, which was generated by transferring a GlcNAc residue using recombinant EXTL3 on GlcUAβ1-3Galβ1-O-C_2H_4NH-benzyloxycarbonyl. Even separately expressed EXT1 or EXT2 alone could form long heparan polymers, which were greater than 1.7°ø10^5 in molecular mass. Moreover, glypican-1, produced by the HS-deficient sog9 cells, could serve as an efficient acceptor for HS polymerization for the EXT1/2 complex, which exhibited much stronger polymerizing activities compared to separately expressed enzymes. Both EXT1 and EXT2 showed weak yet significant polymerizing activity, but lengths of the produced chains were markedly different, sufficiently long HS chains being polymerized by singly expressed EXT1 and much shorter chains in contrast formed by EXT2 alone. These findings suggest that the transfer of the first GlcNAc to GlcUAβ1-3Galβ1-*O*-C_2H_4NH-benzyloxycarbonyl is a rate-limiting step in HS biosynthesis and the

interaction of EXT1 and EXT2 is required for efficient HS polymerization on glypican-1.

(79) ***N*-glycosidase Activity was Found in Commercial Enzyme Preparations**

Atsushi Kobayashi, Noritsugu Daigaku, Masato Noguchi, Masaki Ishihara and Shin-ichiro Shoda

Graduate School of Engineering, Tohoku University, Aoba 6-6-7, Aramaki, Aobaku, Sendai 980-8579, Japan.

Naturally occurring *N*-glycosides appear in nucleic acids, antibiotics, plant metabolites, and glycation products. They have been associated with fundamental roles in many different aspects of biological events. They are biodegradable compounds that are usually decomposed by oxidases or hydrolases. DNA and RNA *N*-glycosylases are well-known enzymes that catalyze hydrolysis of *N*-glycosides. Amadori compounds, one of the intermediate of glycation products, are decomposed by amadoridases. *N*-glycans attached to glycoproteins are cleaved by peptide: *N*-glycosylases, which hydrolyze amide bonds between *N*-acetylglucosamine and aspargine. If certain kind of *N*-glycosidase that hydrolyzes *N*-glycosides exists, this would be a powerful tool for investigating biological phenomenon. In order to seek an activity that hydrolyzes *N*-glycan, we synthesized 1-deoxy-1-*N*-*p*-nitrophenyl β-D-glycosylamine (PNAG) as a screening substrate. A solution of *p*-nitroaniline (10 mmol) in dimethylsulfoxide (16 cm^3) was added to D-glucopyranose (5 mmol), and the mixture was stirred until D-glucopyranose was completely dissolved. The reaction mixture was refluxed for 1 h at 80°C in the presence of acetate (4 cm^3) while stirring. After cooling the reaction mixture to ambient temperature, dropwise addition of toluene precipitated the PNAG as an amorphous powder, yielding pure PNAG (yield: 77%) as a yellow amorphous powder. A reaction mixture (0.2 cm^3) containing 10 mM of the substrate, 15 mM acetate buffer (pH 5.0), and the enzyme preparation (12 mg/cm^3) was incubated at 37°C for 11 hours. Most of commercial cellulase preparations showed the hydrolytic activities, according to the results of the thin layer chromatographic analysis of reaction mixtures. We selected a commercial cellulase preparation from *Trichoderma viride* as a source of *N*-glycosidase, because it showed the highest activity among other enzyme preparations. The catalytic property of the crude enzyme preparation was determined before purification. When PAPG was employed as a substrate, optimum temperature and pH were 90°C and 5.0, respectively. To identify the main component of the *N*-glycosidase activity, chromatographic purifications were employed. These purification steps produced a single band upon SDS-PAGE corresponding to a molecular mass 67kDa. This paper will discuss the enzyme property and substrate specificity of this purified enzyme.

(80) **Elongation of Chitooligosaccharides by Alternative Two-hydrolase Reaction**

Michinari Kohri, Atsushi Kobayashi and Shin-ichiro Shoda

Graduate School of Engineering, Tohoku University, Aoba6-6-7, Aramaki, Aoba-ku, Sendai 980-8579, Japan.

Multiple industrial and medical uses of chitin and its derivatives have been developed in recent years. Especially, water-soluble chitooligosaccharides shows biological activities such as wound healing activity and elicitor activity. However, it is still difficult to prepare chain length-controlled chitooligosaccharides. As a practical approach to obtain chain length-controlled chitooligosaccharides, we have already reported that enzymatic transglycosylating reaction by using oxazoline derivative of *N*-acetyllactosamine (LacNAc-oxa), a transition state analogue for chitinaseA1, as glycosyl donor. This reaction was based on the alternative enzyme reaction with chitinaseA1 from *Bacillus circulans* WL-12 under basic conditions (pH 10.4) and β-galactosidase from *Bacillus circulans* under neutral condition. The main drawback of this reaction was that it was necessary to purify the product after each enzymatic reaction, because two enzymes do not work under same pH condition. In order to overcome the drawback, we attempted two approaches: usage of mutants chitinaseA1 by site-directed mutagenesis and application of a thermostable β-galactosidase. In this study, mutants of chitinaseA1 from *Bacillus circulans* WL-12 were employed as catalyst in order to achieve a one-pot elongation of chitooligosaccharides. We postulated that a tryptophan 433 (W433) plays an important role at the subsite ®C2 for hydrolysis reaction. Mutants chitinaseA1 W433A, W433F, W433H and W433Y were prepared by site-directed mutagenesis, and proteins were produced using *E.coli*. W433Y mutant was the most effective for transglycosylation reaction from LacNAc-oxa to chitobiose in 80% yield, and the transglycosylation product (Gal-GlcNAc-GlcNAc-GlcNAc) was not hydrolyzed; the reaction proceeded irreversibly. The process of GlcNAc unit elongation is as follows: 1) addition of W433Y to a mixture of LacNAc-oxa and chitobiose at 4°C, 2) W433Y-catalyzed transglycosylation reaction at 37°C for 30 min, 3) inactivation of W433Y at 100°C for 5 min, 4) addition of β-galactosidase at 4°C, 5) β-galactosidase-catalyzed degalactosylation reaction at 37°C for 30 min, and 6) inactivation of β-galactosidase at 100°C for 5 min. By using this method, chitotriose was obtained in 74% yield at the first cycle and chitotetraose was obtained in 40% yield at the second cycle. In conclusion, one-pot elongation reaction process of chitooligosaccharides by combined W433Y and β-galactosidase has been achieved.

(81) **Metabolically Engineered Agrobacterium sp as Whole-Cell Biocatalysts in Oligosaccharides Synthesis**

Rachel R Chen and Zichao Mao

School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA 30332-0100.

Carbohydrate moieties of glycoconjugates play key roles in cell adhesion, inflammation, cancer, metastasis and many other disease-causing events. Carbohydrate-based molecules are being pursued by many pharmaceutical and biotech companies as anti-infective drugs and vaccines. But the synthetic difficulties in generating these molecules with the required specific linkage and anomerity have long hampered the clinical development of carbohydrate-based therapy. Enzyme-based strategies toward complex carbohydrate represent emerging technologies that have the potential to greatly simplify synthesis process. However, the obstacle in using glycosyltransferase enzymes for synthesis is the cofactors requirement (sugar nucleotides), which are expensive and not readily available.

By resorting to nature's biodiversity, we are developing an efficient whole-cell based sugar nucleotide regeneration system. *Agrobacterium* sp ATCC 31749 is an industrial strain used to produce a homo glucose polymer called curdlan. Curdlan synthesis in this strain is very efficient, with productivity 1.0 g-curdlan/hr/g-dry cell and yield of 0.5 g curdlan/g glucose and final concentration reaching 60 g/l. The extraordinarily high productivity of the glucose polymer in this strain implicates an efficient UDP-glucose cofactor regeneration mechanism, which in turn can be attributed to the exquisite mechanism that couples the energy production, carbon metabolism and UDP-glucose synthesis.

In order to harness the efficient cofactor regeneration mechanism, we used a mutant of ATCC 31749 in which the polymerization of the precursor molecule (UDP-glucose) catalyzed by curdlan synthase was disrupted with a miniTn5lacZ1(Km^R) transposon mutation, resulting in a 80% reduction in curdlan synthesis. The mutants were found to maintain comparable intracellular concentration of UDP-glucose as compared to the wild type cells. Further metabolic engineering was accomplished by heterologous expression using a broad host-range expression vector. Functional expression of *E. coli* UDP-galactose-4- epimerase (catalyzing the conversion between UDP-glucose and UDP-galactose) in the curdlan deficient strain was achieved, increasing the epimerase activity approximately 10 fold. Thus the engineered *Agrobacterium* strain could effectively function as whole-cell UDP-glucose and UDP-galactose regeneration systems, making it useful in enzymatic synthesis of glucose and/or galactose-containing oligosaccharides as an in-situ cofactor regenerator. We have demonstrated utility of such system in the synthesis of a model compound, N-acetyllactosamine (a disaccharide consisting of galactose and N-acetylglucosamine).

Our continued efforts in enhancing the utility of the developed cofactor regeneration systems include using fusion expression to combine the cofactor regeneration system with requisite glycosyltransferases for oligosaccharide synthesis. We are also extending the application of the natural UDP-glucose regeneration system to other sugar nucleotides such as UDP-glucuronic acid, UDP-galactouronic acid. Thus the metabolically engineered *Agrobacterium* cells could potentially provide four common sugar precursors that appear in most human glycans.

(82) **Biochemical and Structural Studies on Two Glucuronyltransferases Involved in the Biosynthesis of HNK-1 Carbohydrate Epitope**

Shinako Kakuda[1,3], Tomoo Shiba[2], Yasuhiro Tonoyama[1], Yasuhiko Kizuka[1], Shogo Oka[1],
Soichi Wakatsuki[2], Ryuichi Kato[2] and Toshisuke Kawasaki[1]

[1] Dept. of Biol. Chem., Grad. Sch. of Pharm. Sci.,Kyoto Univ., [2] Struct. Biol. Res. Center, KEK, Dept. of Biol. Chem., [3] 21Centry, COE.

The HNK-1 carbohydrate epitope is found on some neural cell adhesion molecules. The characteristic structure of this epitope is sulfated glucuronic acid attached to *N*-acetyllactosamine structure, HSO_3-3GlcA-Galß1-4GlcNAc. Two glucuronyltransferases, GlcAT-P and GlcAT-S, are essential enzymes involved in the biosynthesis of the HNK-1 epitope. We expressed and purified from COS-1 cells, and their properties and acceptor specificities were clarified. Both GlcAT-P and GlcAT-S transferred a glucuronic acid not only to a glycoprotein acceptor, asialo-olosomucoid, but also to a glycolipid acceptor, paragloboside. GlcAT-P specifically recognized *N*-acetyllactosamine (Galβ1-4GlcNAc) structure at the non-reducing terminal of acceptor substrates. In contrast, GlcAT-S recognized not only the terminal Galβ1-4GlcNAc structure but also Galβ1-3GlcNAc and Galβ1-4Glc structures and showed the highest activity to triantennary *N*-linked oligosaccharides.

To investigate the substrate specificity and the structure and function relationship of these enzymes, large amounts of soluble forms of human GlcAT-P and GlcAT-S from *E. coli.* were prepared. Using the recombinant GlcAT-P purified form *E. coli*, we determined the X-ray crystal structures. We found that the donor nucleotide sugar is recognized by conserved amino acid residues including DXD motif (Asp195, Asp196, Asp197). In addition, Val320 and Asn321, which are located on the C-terminal loop in a neighboring molecule, and Phe245 contribute to the interaction with GlcNAc moiety of *N*-acetyllactosamine of acceptor substrate. The unique combination of the aromatic interaction of Phe245, the hydrophobic interaction of Val320 and the hydrogen bond of Asn321, which are characteristic in GlcAT-P, play a key role in establishing the acceptor substrate specificity. Based on the structural and biochemical results, we will discuss the molecular mechanisms of substrate recognition of GlcAT-P and GlcAT-S.

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(83) A Practical Synthesis of Cyclodextrin-Scaffolded Glycoclusters (GlycoCDs) Based on the Combined Chemical and Enzymatic Modifications

Tetsuya Furuike[1], Nobuo Sakairi[1] and Shin-Ichiro Nishimura[2,3]

[1] Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan, [2] Division of Biological Sciences, Graduate School of Science, Frontier Research Center for Post-Genomic Science and Technology, Hokkaido University, Sapporo 001-0021, Japan, [3] Research Center for Glycoscience, National Institute of Advanced Industria

Science and Technology (AIST), Sapporo 062-8517, Japan.

Å@Å@Carbohydrates existing on cell surface play important roles in numerous intercellular recognition processes. Interactions between carbohydrates and carbohydrate-binding proteins are crucial steps of the specific biological processes such as cell differentiation, immune response, infection, and cancer metastasis. Although a variety of oligosaccharides as partial structures of glycolipids, glycoproteins, and proteoglycans serve significant biological signals and they can be used as simple inhibitors for testing the glucoconjugates functions, it has been reported that low affinity and broad specificity are often observed in the interactions of these oligosaccharide-based ligands with lectins. Therefore, it might be indispensable to investigate how such weak and vague interactions can be amplified or controlled in the successful processes for the sugar-recognition and the signal transduction. The glycoside cluster effect proposed by Y. C. Lee has attracted considerable attention and promoted extensive efforts by synthetic chemists to design a variety of multivalent glycoligands. Cyclodextrin-based glycoclusters as well as polymers, dendrimers, calix[4]allenes and peptides are useful tools and reagents to investigate the significance of the multivalency in the carbohydrate-protein interactions. Moreover, macrocyclic carbohydrateclusters are strongly expected to be specific molecular transporters to the carbohydrate binding proteins existing on the cell surfaces. Here, we describe a simple and highly practical method for the synthesis of cyclodextrin-scaffolded glycoclusters (glycoCDs).

Å@Å@First, the coupling reaction of heptakis 6-deoxy-6-iodo-β-cyclodextrin (6-I-CD) with unprotected sodium thiolates derived from the corresponding thioacetylalkyl glycosides proceeded smoothly in mild condition, and gave these corresponding glycoCDs having galactose (Gal), *N*-acetyl-D-glucosamine (GlcNAc), lactose (Lac), and *N*-acetyllactosamine (LacNAc) residues in high yields (78-88%), respectively.[1] However, the direct introduction of more complicated carbohydrate structure onto the CD scaffold was very difficult for the steric hindrance of itself. Therefore, we suggested a synthetic strategy for the glycoCDs based on the combined chemical and enzymatic modifications. Thus, a key intermediate, the glycoCD having seven GlcNAc residues (GlcNAcCD) as the minimum sugar unit, was chemically prepared according to the procedure as described above. Next, enzymatic galactosylation using UDP-Gal with a bovine milk β-1,4-galactosyltransferase and subsequent sialylation with a recombinant α-2,3-sialyltransferase in the presence of CMP-Neu5Ac proceeded smoothly and gave a mono-dispersed glycoCD that completely substituted with seven SialylLacNAc branches at C-6 positions in excellent overall yield. Moreover, to expand the potential of glycoCDs as practical therapeutic reagents, our attention has been focused on the synthesis of the glycoCD having more complicated oligosaccharide branches by means of the above chemo-enzymatic strategy. Thus, the glycoCD having twenty-one GlcNAc residues (TrisGlcNAcCD) was efficiently prepared from the nucleophilic substitution reaction of 6-I-CD with trivalent GlcNAc derivative having sodium thiolate at the non-reducing end. As the result, the subsequent stepwise elongations of the TrisGlcNAcCD proceeded by two above glycosyltransferases and allowed to give the desired glycoCD having twenty-one SialylLacNAc residues in excellent yield. Å@Å@In conclusion, we have established an efficient and practical method for the synthesis of the glycoCD having seven or twenty-one SialylLacNAc branches by means of the combined chemical and enzymatic strategy.

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(84) Efficient Production of GM3-type Oligosaccharide by Using Cells

Yoshimi Murozuka, Maria Carmelita Kasuya and Kenichi Hatanaka

Institute of Industrial Science, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan.

The usual synthetic methods for the preparation of oligosaccharides require stepwise elongation. For example, chemical synthesis remains to be a tedious task requiring intricate methods which consist of protection, glycosylation, and deprotection steps. Thus, we employed biocombinatorial synthesis of oligosaccharides using primers and cells. Saccharide primers are synthetically accessible amphiphilic glycoside derivatives that resemble intermediates in the biosynthetic pathway of glycolipids and act as substrates for cellular enzyme-catalyzed glycosylation. In order to establish the optimum condition for the bio-production of a large amount of valuable materials containing GM3-type oligosaccharides, two kinds of lactoside primers having azido group at different positions were synthesized and introduced into B16 melanoma cells. The synthesis of primersáT and áU was accomplished by glycosylation of 12-azido-1-dodecanol and 2-azido-1-dodecanol, respectively, with lactose peracetate and subsequent deacetylation. Both primers were glycosylated to give GM3-type oligosaccharide derivatives which were released to the culture medium. The amount of glycosylated product from primeráU (2-azidododecyl β-lactoside) was almost twice as compared with that from primeráT(12-azidododecyl β-lactoside). The effects of seeded cell number, primer concentration and length of incubation time on the glycosylation efficiency were also investigated. The results are necessary to optimize the conditions for the mass-production of GM3-type oligosaccharide using azido dodecyl lactoside primer and B16 cells.

(85) Fluorous-Tagged Saccharide Primers for Oligosaccharide Synthesis Using Cells

Maria Carmelita Kasuya, Ayaka Ito, Reuben Cusi, Orie Ishihara and Kenichi Hatanaka

Institute of Industrial Science, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan.

Facile synthesis of oligosaccharides could be accomplished by combining chemical synthetic methods with cellular biosynthetic processes [1]. This strategy involves the preparation of saccharide primers (amphiphilic glycoside derivatives) by simple synthetic means, and subsequent incorporation of these primers into cells that serve as substrates for glycosylation by cellular enzymes. The cellular uptake of primers and release of glycosylation products to the culture medium by cells depend not only on the saccharide moiety and number of hydrophilic groups, but also on the hydrophobic aglycon unit.

Fluorinated compounds can also act as viable building blocks for oligosaccharide synthesis by cellular enzymes [2]. In this research, the incorporation of fluorine to the lipophilic aglycon unit of the lactoside primer was carried out to further establish the role of the aglycon unit in priming oligosaccharide synthesis. Moreover, the incorporation of a fluorous tag is perceived to facilitate the extraction of the glycosylated product from the culture medium by using a fluorous solvent.

Lactoside **(Lac)**, galactoside **(Gal)** and glucoside **(Glu)** primers with different fluorous-tags, perfluorohexylhexyl **(F6)** or perfluorodecylethyl **(F10)**, were prepared and were examined in mouse B16 melanoma cells for their feasibility as substrate for oligosaccharide biosynthesis. The synthesis of the fluorous-tagged lactoside primers was accomplished by glycosylation of lactose peracetate with perfluorohexylhexanol, or with perfluorodecylethanol, followed by deacetylation. Synthesis of the fluorous-tagged galactoside and fluorous-tagged glucoside primers was carried out in a similar manner.

After 48-h incubation of cells with the primer, the lipids were extracted from the cell homogenates and the culture media and analyzed by HPTLC. New bands were extracted from the HPTLC plate and analyzed by MALDI TOF mass spectrometry. Results showed that incorporation of the lactoside primers **(Lac-F6 or Lac-F10)** gave monosialylated products. Treatment of the glycosylated products with α-(2→3) neuraminidase from *Arthrobacter ureafaciens* in phosphate buffer (pH 7.3) for 16 h at 37 °C confirmed that the product is α-(2→3) sialylated lactoside which is the same oligosaccharide as GM3 - the glycosphingolipid predominantly expressed on the cell surface of B16 melanoma cells, and effective on several important bioactivities. Treatment of B16 cells with the galactoside primer **(Gal-F6)** likewise gave a monosialylated product. On the other, **Gal-F10** primer and the glucoside primers **(Glu-F6 and Glu-F10)** were not glycosylated.

This research demonstrates for the first time that fluorous-tagged compounds could be taken-up by the cell and take part in the biosynthetic machinery to afford the sialylated oligosaccharides without the need for a series of protection and deprotection steps usually required for chemical synthesis. The efficient extraction of the sialylated products with fluorous solvents is in progress.

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(86) Facile and Library-Oriented Methodology for Solution Phase Oligosaccharide Synthesis

Maki Takatani[1,2] and Yukishige Ito[1,2]

[1] RIKEN (The Institute of Physical and Chemical Research), [2] CREST, JST (Japan Science and Technology Agency), Japan.

N-linked oligosaccharide moieties of glycoprotein play a role as recognition signal in many biological events. For instance, endoplasmic reticulum (ER) residing molecular chaperones calnexin (CNX) and calreticulin (CRT) are considered to recognize the oligosaccharide portion ($Glc_1Man_9GlcNAc_2$) of glycoproteins and assist their folding. Subsequently, terminal glucose (Glc) is removed by glucosidase II and glycoproteins carrying undecasaccharide ($Man_9GlcNAc_2$) are transported to Golgi for further processing. Other major players in glycoprotein quality control are glucosyl transferase (UGGT), mannosidase-like lectin (EDEM), cargo receptors (VIP36, ERGIC-53) and ubiqutin ligase (Fbx2). All of these proteins likely recognize precisely different oligosaccharide structures, although the precise picture of these phenomena is unclear. As the first step to gain clear understanding of these processes, we achieved the first chemical synthesis of dodecasaccharide ($Glc_1Man_9GlcNAc_2$)[1], which is a putative ligand of CNX and CRT. In order to gain more precise understanding on the structural requirement for the recognition of CRT/CNX and other lectins involved in glycoprotein quality control, systematic preparations of various high-mannose glycan chains and their partial structures are required.

We wish to report an efficient synthetic strategy of ER-related oligosaccharides and their partial structures. The synthesis was designed to be purification-free and applicable to library construction. It commenced with the dimannoside derivative having lipophilic tether which had been obtained by *p*-methoxybenzyl assisted intramolecular aglycon delivery[2] and subsequent glycosylation. Thioglycoside, which has a *tert*-butyl-dimethylsilyl (TBDMS) group for temporary hydroxy protection, was employed as a first glycosyl donor. Coupling of these compounds was promoted by methyl triflate (MeOTf) and di-*tert*-butyl-4-methylpyridine (DTBMP) in toluene to give trisaccharide. After acetylation of unreacted acceptor, desilylation was achieved by treatment with tetrabutylammonium fluoride. For the second glycosylation, pentafluoropropionyl (PFP)[3], protected mannosyl fluoride was used as the donor and was activated with $AgClO_4$-Cp_2HfCl_2 -DTBMP to give tetrasaccharide. The crude mixture was capped by acetylation again, subsequent deprotection of *O*-PFP was performed using pyridine-MeOH. The final glycosylation with chloroacetyl (CAc) protected thioglucoside was effected by MeOTf-DTBMP in toluene. Thus obtained crude mixture, which contained not only pentasaccharide but also deletion products (tetra-, tri-, disaccharides), was subjected to the capture-release purification strategy[4]. Namely the products, carrying a CAc group, were captured by resin bound Boc protected cysteine and released by successive treatment with TFA and piperidine. The release products were fully deprotected, passed through Sep-Pak C_{18} cartridge to give the target pentasaccharide. On the other hand, the residue, which wasn't capture by resin, was purified by size exclusion chromatograohy to afford the tetra-, tri-, and disaccharides. These obtained compounds are partial structures related to glycoprotein quality control. With combined use of volatile protecting group (e.g. TBDMS, PFP), CAc (for capture-release), and hydrophobic tag, facile and purification-free synthesis of oligosaccharide was achieved. Potential application to the library construction will be discussed.

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(87) Chemoenzymatic Synthesis of Glycoconjugate Polymers Starting from Non-reducing Disaccharides

Natsuko Wada, Yoshiko Miura, Yoshihiro Nishida and Kazukiyo Kobayashi

Department of Molecular Design and Engineering, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8603, Japan. .

Carbohydrates, widely expressed on cell surfaces, play important roles in a variety of biological. Synthetic glycoconjugates are important as tools to investigate glycobiological phenomena including carbohydrate-protein interactions and also as anti-infective agents, synthetic antibodies, and biosensors to apply the recognition events. Glycoconjugate polymers are paid much attention because of large glycocluster effects. A number of glycoconjugate polymers with styrene and acrylamide backbones were reported to show outstanding biological functions. In addition, various research collaborations have developed biomedical, pharmaceutical and medical applications, such as tissue engineering, and dug delivery. Biological safety including biocompatibility, biodegradability, and bioabsorbability and non-cytotoxity is also important in biomedical applications of glycoconjugate polymers. We have synthesized glycopolymers with poly(vinyl alcohol) backbone via a chemoenzymatic procedure. The poly(vinyl alcohol) backbone is known to have the advantage of biocompatibility and biodegradability. Moreover, the chemoenzymatic access to glycoconjugate polymers is favorable from an environmental view point. Here, we applied the chemoenzymatic synthesis of glycoconjugate polymers to two non-redusing disaccharide: trehalose (Glcα1-1αGlc) and Gal-type trehalose (Galα1-1αGlc). Because of the stereochemical similarity of Gal-type trehalose to globosyl Gb2 and Gb3, the polymer with Gal-type trehalose is a potential ligand of Shiga toxins (Stxs). The unique chemical structure and property of trehalose also attract the interest for the biological application of the glycopolymer with trehalose. Chemoenzymatic synthesis was carried out through esterification of disaccharides and the subsequent radical polymerization. Esterification of trehalose and Gal-type trehalose was screened using several kinds of lipases. Lipases from *Pseudomonas cepacia* showed high activity to both disaccharides to afford the disaccharide vinyl esters in modest yields. The disaccharide vinyl esters were polymerized with an initiator H_2O_2/ascorbic acid to yield the glycoconjugate polymers. Lipase catalyzed esterification was completely selective at the primary Glc 6-OH position of trehalose and Gal 6-OH of Gal-type trehalose. The biological ability of the glycoconjugate polymers were analyzed with FITC-labeled lectins: α-Glc supecific Con A, α-Gal specific *B.Simplicifolia*, and β-Gal specific RCA_{120}. Trehalose polymer showed the highest activity to Con A due to the glyco-cluster effects and the dimeric α-Glc structure. As well, the Gal-type trehalose polymer binds to *B.Simplicifolia* effectively. The glycoconjugate

polymers showed no apparent affinity to RCA_{120} in spite of Gal moiety. The Stx-1 binding assay was carried out using self-assembled monolayer of Gb3 (Gb3-SAM) on quartz crystal microbalance (QCM). The notable inhibitory activity to the adhesion of Stx-1 to the Gb3-SAM was observed with the addition of the Gal-type trehalose polymer into the Stx-1 solution. Miura, Y.; Wada, N.; Nishida, Y.; Kobayashi, K. J. Polym.Sci.A, 2004, in press. Miura, Y.; Ikeda, T.; Kobayashi, K. Biomacromolecules, 2003, 4, 410.

(88) **A Synthesis of UDP-Oligosaccharides**

Hironao Takaku[1,2], Hide-ki Ishida[1], Toshiyuki Inazu[1,3], Hideharu Ishida[2] and Makoto Kiso[2]

[1] The Noguchi Institute, 1-8-1 Kaga, Itabashi-ku, Tokyo 173-0003, Japan, [2] United Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, Japan, [3] Department of Applied Chemistry, School of Engeneering, Tokai University, Kitakaname 1117, Hiratsuka, Kanagawa 259-1292, Japan.

The natural synthesis of poly- or oligosaccharides is performed in a stepwise manner by glycosyltransferases that use sugar nucleotides such as UDP-GlcNAc, GDP-Man and CMP-Neu5Ac as glycosyl donors. By the way, in 1960's some UDP-oligosaccharides were isolated from milk of mammals, including human, and characterized as elongated UDP-*N*-acetylglucosamine derivatives. Although these UDP-oligosaccharides are known to be present in higher animals, their biosynthetic pathways and biological functions such as activities as glycosyl donors have not yet been elucidated because of their limited quantities. In order to elucidate the functions of UDP-oligosaccharides a variety of natural-type compounds and their analogs are required. Enzymatic syntheses of UDP-*N*-acetyllactosamine using galactosyltransferase or galactosidase were reported[1], but it is difficult to prepare many kind of compound including analogs by enzymatic methods because of the restriction of substrate specificity. On the other hand, chemical method enables to synthesize many kinds of compounds and analogs, once oligosaccharide moieties are prepared. So, we describe herein a chemical synthesis of UDP-*N*-acetyllactosamine and UDP-lactose for searching glycosyltransferases that use these UDP-oligosaccharides as glycosyl donors and that transfer the oligosaccharides moiety to glycosyl acceptors. For the synthesis of UDP-oligosaccharides, 1-phosphates of oligosaccharides were prepared by coupling of protected *N*-acetyllactosaminyl trichloroacetimidate[2] with dibenzylphosphate or coupling of unprotected 3-methoxy-2-pyridyl (MOP) lactoside with phosphoric acid[3], respectively. Then condensation of these 1-phosphates of oligosaccharidesÅ@and UMP-imidazolate[4] gave the corresponding UDP-oligosaccharides after purification by column chromatography of anion exchange resin and gel filtration.

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(89) **Synthesis of the Terminal Pentaarabinofuranoside Derivatives of Cell Wall Skeleton from *Mycobacterium tuberculosis***

Hiroko Akao, Akihiro Ishiwata and Yukishige Ito

RIKEN (The Institute of Physical and Chemical Research).

The *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) strain is a tuberculosis vaccine strain, which is almost nonpathogenic and contributed to decrease in this disease and whose cell wall skeleton is known as an effective immune adjuvant.[1)] However, the detailed mechanisms of BCG-CWS to potentiate the immune system is not clear and any appropriate conception has not been obtained about the relationship between the constituents of BCG-CWS and the biological activities.

BCG-CWS consists of mycolic acid, D-arabinan, D-galactan, linker disaccharide (α-Rham-(1→3)-α-GlcNAc), and peptidoglycan (PG) which is composed of GlcNAc-Mur(*N*-glycolic). Uniquely, all of the arabinose and galactose is present in the furanose form. Their nonreducing termini are decorated by pentasaccharide motifs, each of which is esterified at the four primary hydroxyl groups with long chain branched β-hydroxylcarboxylic acids (mycolic acids) .

We are interested in the terminal lipo-pentaarabinofuranoside, which is close to the surface of the cell and perceived to be involved in a series of immunological events after infection. Here we wish to report the synthesis of this moiety in order to examine its biological activity.

Our targets are the derivatives of terminal pentaarabinofuranoside, β-D-Ara*f*-(1→2)-α-D-Ara*f*-(1→3)-[β-D-Ara*f*-(1→2)-α-D-Ara*f*-(1→5)]-D-Ara*f*. Lowary *et al*. already reported the synthesis of the same sequence of this oligosaccharide moiety.[2)] In our case different protecting groups of donors and acceptors were used in order that further modification of terminal arabinose would be possible. Synthetic scheme was planned and carried out as follows. 1) It commenced with the preparation of the core trisaccharide moiety, which was gained in good yield by selecting appropriate activating agents. 2) Then the construction of the pentasaccharide was attempted by stereoselective β-glycosylation using selectively protected arabinofuranose donors.

For this purpose, several donors with various protecting groups at C5 position were examined. Furthermore, reaction conditions, for example temperature, solvent and so on were devised. Through this optimization process, α/β ratio of these glycosylation steps was found to be influenced by the protecting groups at C5 position. It was found that the use of electron donating protecting groups somewhat facilitates β-glycosylation. According to this result the preparation of the pentasaccharide derivative was pursued.

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(90) **Combinatorial Synthesis of DTPA-Conjugated Oligosaccharides as MRI Contrast Agents**

Yoshio Ando[1], Hiroshi Tanaka[1], Masatoshi Wada[2] and Takashi Takahashi[1]

[1] Department of Applied Chemistry, Graduate School of Science and Engineering, Tokyo Institute of Technology, 2-12-1 Ookayama, Meguro, Tokyo 152-8552, Japan, [2] Nihon Medi-physics, 3-1, Kitasode, Sodegaura-Shi, Chiba 299-026, Japan.

Oligosaccharides are important skeletal material in plants and crustaceans, and have served as effective biocompatible materials in medical fields such as biomedicine, pharmacology and biotechnology. Chitosan and its hydrosates are specially effective as the biocompatible scaffolds, which can be conjugated with various functional molecules through amide bonds.
 Magnetic resonance imaging (MRI) is a powerful and noninvasive diagnostic technique useful in providing images of the inside of the human body. MRI contrast agents are exogenous compounds able to enhance the relaxation rates of water protons, and should show low toxicity. Recently, CH3-DTPA-Gd composed of chitotritol and gadolinium-diethylenetriamine-N,N,N',N'',N''- pentaacetic acid complexes (Gd-DTPA) has been reported as a new class of MRI contrast agent. The relaxivity per Gd of CH3-DTPA-Gd is R_1 = 8.3 $(mMs)^{-1}$ at 37 □C in water at 1.5 T, compared to Gd-DTPA with R_1 = 3.1 $(mMs)^{-1}$. The saccharide scaffold with CH3-DTPA-Gd would be effective to increase the relaxation rate ($1/T_1$) of water protons by the Gd complex without toxicity. However, influence of the variation of the saccharide scaffold on the relaxation rate is unclear because the starting material is originated from chitosan. Additionally, in order to use such glycoconjugates for clinical propose, elucidation of the function and toxicity of minor impurity would be important. Therefore, an effective methodology for the synthesis of such glycoconjugates varying the saccharide backbone is required. Herein we descried the combinatorial synthesis of DTPA-conjugated oligosaccharides varying their stereochemistry of the anomeric positions.
 We planned the synthesis of 14 DTPA-conjugated oligosaccharides involving 8 trisaccharides, 4 disaccharides and 2 monosaccharides varying the stereochemistry at the anomeric position and C2 position on reducing end. Our strategy for the synthesis of the glycoconjugates involves preparation of the corresponding protected oligosaccharides, followed by conjugation with DTPA unit and deprotection. We designed perbenzyl and perazido protected oligosaccharides. These protecting groups can be removed by evaporable or filterable reagents, which would be effective for purification of the hydrophilic compounds. Additionally, protected oligosaccharides possessing same protecting groups would be easily converted to the DTPA-conjugated oligosaccharides in parallel fashion.
 Preparation of the key intermediates was examined. In optimization of reaction conditions by the solvent successfully resulted in the yield of a- and b- mixture of glycosides (a:b = 1:1) from 2-azido glycosyl imidates. Separation of the each isomers was achieved by column chromatography on silica gel to provide two pure stereoisomers. Using 4 building blocks, 14 tri- to mono-saccharides were prepared. Reduction of the azido groups by trimethylphosphine provided

the corresponding amines, followed by acylation of the amine with tetra-t-butyl protected DTPA mono acid. Finally, deprotection of the t-butyl esters and benzyl protecting groups afforded the 14 DTPA-conjugated oligosaccharides.
 The effects of the synthesized DTPA-conjugated oligosaccharides on signal enhancement ability per Gd ion were tested by MRI phantom study. DTPA was used as a reference ligand for the MRI study. Trisaccharide derivatives showed stronger ability than mono- and di-saccharides. There are few effects of the stereochemistry of the saccharide scaffold on the increasing ralaxation rate of water proton.

(91) ***In Situ*** **Chemoenzymatic Synthesis of Solid-Phase Glycosaminoglycan Oligosaccharide Libraries and Probing Protein Binding Specificity**

Breca S Tracy and Paul L DeAngelis
University of Oklahoma Health Sciences Center, Department of Biochemistry and Molecular Biology, Oklahoma Center for Medical Glycobiology, 940 S.L. Young Blvd, Oklahoma City, OK 73104.

Glycosaminoglycans (GAGs), long polysaccharide chains composed of repeating disaccharide units containing a hexosamine, and various binding proteins play key roles throughout the vertebrate body such as mediating and/or regulating adhesion, signaling, coagulation and inflammation. The extracellular matrix and cell surfaces contain various GAGs such as hyaluronan (HA) and chondroitin (C). Multiple interactions occur between proteins and HA and C, but their exact binding specificities remain unknown. We are now determining binding specificity utilizing solid-phase GAG oligosaccharide libraries made by *in situ* chemoenzymatic synthesis. Short sugar chains composed of hyaluronan, chondroitin or combinations of both GAGs (5-20 monosaccharides in length) were synthesized in a stepwise fashion in a 96-well plate format using the *Pasteurella* catalysts, hyaluronan synthase (pmHAS) and chondroitin synthase (pmCS). These two enzymes enable us to create a unique library of hybrid oligosaccharides varying in both composition and length (e.g. HA4-C2, C4-HA2, C7, HA13) that can then be probed with various proteins. Rapid, stepwise synthesis of oligosaccharides takes place in aqueous buffers (approximately one sugar addition step in thirty minutes total time). The GAG oligosaccharide libraries were probed with proteins including aggrecan fragments and tumor necrosis factor stimulated gene-6 (TSG-6). Binding was either detected colorimetrically with enzyme-linked conjugates or with fluorescence readout. The GAG oligosaccharide libraries are conveniently reusable (may be reprobed at least three times). The general format should also be compatible with cell-based assays. Our preliminary results indicate that GAG binding specificity of the various proteins depends on the length and the composition of sugar chains. Therefore, the development of more potent and/or selective ligands for the binding proteins should be possible. Hybrid oligosaccharides and oligosaccharides of specific length should be beneficial for future medical uses such as tissue engineering biomaterials and novel therapeutics (e.g. anti-cancer agents and angiogenesis modulators). (Supported by National Science Foundation and Oklahoma Center for Advancement of Science and Technology)

(92) **M6749 Antibody, Which has Similar Specificity to HNK-1 Antibody, Recognizes a Subpopulation of Mouse Lymphocytes**

Yukina Kiyama[1,2], Toshikazu Jinnouchi[1], Shogo Oka[1,2] and Toshisuke Kawasaki[1]
[1] Dept. of Biol. Chem., Grad. Sch. of Pharm. Sci., Kyoto Univ., [2] CREST.

The HNK-1 carbohydrate is well known to be expressed in the human immune system as well as in the nervous system. The structure of the carbohydrate is the sulfated glucuronic acid attached to *N*-acetyllactosamine (HSO_3-3GlcAβ1-3Galβ1-4GlcNAc). The HNK-1 carbohydrate in the nervous system has been shown to be involved in higher ordered brain functions including learning and memory. However, little is known about the role of the HNK-1 carbohydrate in the immune system and even the question whether or not the HNK-1 carbohydrate is expressed in the immune system is not clear other than the human immune system. In this study, we examined the expression of the HNK-1 carbohydrate on mouse immune cells to elucidate the role of the HNK-1 carbohydrate in the immune system. Single cell suspensions were prepared from 8-10 weeks old mouse spleen. After lysis of erythrocytes, lymphocytes were isolated by using Ficoll-sodium diatrizoate solution. Then, the isolated lymphocytes were applied to nylon wool column to remove B cells. The nylon wool-nonadherent lymphocytes were first incubated with HNK-1 mAb, which recognizes sulfated HNK-1 carbohydrate, or M6749 mAb, which recognizes non-sulfated HNK-1 carbohydrate as well as sulfated HNK-1 carbohydrate. After three washes, the lymphocytes were incubated with the FITC-conjugated anti-mouse IgM antibody and analyzed by flow cytometry. About 15-20% of the lymphocytes bound to M6749 mAb, whereas essentially no lymphocytes reacted with HNK-1 mAb. This immunoreactivity is inhibited by the addition of the chemically synthesized HNK-1 carbohydrate (HSO_3-3GlcAβ1-3Galβ1-4GlcNAc). These data indicated that the carbohydrate recognized by M6749 mAb, but not by HNK-1 mAb, was expressed on a subset of mouse lymphocytes. In addition, the subpopulation of the cells recognized by M6749 mAb also bound to anti-NK1.1 mAb, a natural killer (NK) cell marker, suggesting that the carbohydrate recognized by M6749 mAb were expressed on NK cells as well as on the other subset of the lymphocytes besides NK cells. This is the first evidence that the carbohydrate which reacts with M6749 mAb, but not with HNK-1 mAb, is expressed in the mouse immune system.

(93) **Identification Corneal Keratan Sulfate Oligosaccharides by Electrosprary Ionization Tandem Mass Spectrometry**

Yuntao Zhang[1], Yutaka Kariya[2], Abigail H. Conrad[1], Elena S. Tasheva[1] and Gary W. Conrad [1]
[1] Division of Biology, Kansas State University, Manhattan, Kansas 66506, [2] Central Research Laboratories, Seikagaku Corporation, Higashiyamato-shi, Tokyo 207-0021, Japan.

In this study, electrospray ionization tandem mass spectrometry (ESI-MS/MS) was employed to identify corneal keratan sulfate oligosaccharides. In MS1 spectra of standard mono, di-, tri-, and tetra-sulfated KS oligosaccharides, the charge state of the most abundant molecular ion equals the number of sulfate groups in the oligosaccharide. MS2 and MS3 spectra of the variously charged MS1 ions reveal diagnostic fragment ions as fingerprint maps to be used to determine the sequence of an unknown KS hexamer mixture isolated from shark cartilage KS. The component oligosaccharides of an enzyme digest of bovine corneal KS were identified by ESI-MS/MS without chromatography pre-separating. Key words: keratan sulfate oligosaccharides, electrosprary ionization tandem mass spectrometry

(94) **Expression of the HNK-1 carbohydrate Epitope Without Sulfate in Mouse Kidney**

Hideki Tagawa[1,2], Tomoko Ikeda[1], Daisuke Anzai[1], Nana Kawasaki[3], Shogo Oka[1,2] and Toshisuke Kawasaki[1]
[1] Department of Biological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Japan, [2] CREST, [3] Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Japan.

The HNK-1 carbohydrate epitope, which is recognized with a monoclonal HNK-1 antibody, is a unique modification of glycans. The characteristic structure of the HNK-1 carbohydrate epitope is 3-sulfo-glucuronyl residue attached to lactosamine structures (Galβ1-4GlcNAc) on a series of cell adhesion molecules and on some glycolipids. The epitope is expressed mostly in the nervous system over a wide range of species and its expression is spatially and temporally regulated during the development of the nervous system. We have cloned two glucuronyltransferases (GlcAT-P and GlcAT-S) and one sulfotransferase (HNK-1 ST), which are responsible for the biosynthesis of this epitope. During the course of a study using these molecular tools, we found interesting observations. Thus, GlcAT-P mRNA was expressed almost exclusively in brain. In contrast, GlcAT-S mRNA was expressed higher in kidney than in brain. However HNK-1 ST mRNA was expressed almost ubiquitously among various tissues, it was not expressed significantly in kidney. These results suggested that the HNK-1 carbohydrate epitope without sulfate is expressed in kidney. To test this, we used two different monoclonal antibodies, HNK-1 and M6749. HNK-1 mAb requires sulfate on the glucuronyl residue for its binding and M6749 mAb does not. Western blot analysis of the membrane fractions prepared from mouse kidney, two major bands were detected with M6749 mAb (one at the position of 80kDa and the other at 140kDa), while no positive band was detected with HNK-1 mAb. Both of the positive bands detected with M6749 mAb disappeared completely after digestion with *N*-glycanase F, suggesting that these proteins bear the HNK-1 carbohydrate epitope without sulfate on *N*-glycan chains. The following proteomics-type analysis using LC/MS identified that 80kDa band and 140kDa band are meprin α subunit and CD13/Aminopeptidase N, respectively. Both proteins are zinc metalloproteases with 10 potential *N*-glycosylation sites. Immunohistochemical staining of mouse kidney revealed that the HNK-1

carbohydrate without sulfate was expressed in the proximal tubules but not in distal tubules and glomeruli.

(95) **Toward Synthesis of Complex Type *N*-glycans of Helminth Origin**

Jun Nakano[1,2], Hiromichi Ohta[2] and Yukishige Ito[1,3]
[1] RIKEN (The Institute of Physical and Chemical Research), [2] Keio University, [3] CREST (JST).

Recent investigation suggested that the complex type *N*-glycans containing β1-2 xylose and/or α1-3 fucose have strong antigenecity[1]. The glycoproteins produced by plants as well as parasites have these structures. Interestingly, it has been revealed that the human infected with the parasite has tolerance to allergy[2]. In order to understand the relationship between infection of parasites and allergy, synthesis of the saccharide found in parasites and investigation of its function are desired. To that end, we started the investigation toward synthesis of complex type *N*-glycan ($GlcNAcMan_3XylFuc_2GlcNAc_2$) found in egg of parasites, *Schistosome mansoni* and *Schistosome japonicum*. This glycan is also interesting from synthetic point of view, because of its multiply branched structure that consist of a variety of sugar residues.

This nonasaccharide retrosynthetically disconnected to fragment hexasaccharide (fragment **A** : $GlcNAcMan_3XylGlcNAc$) and trisaccharide (fragment **B** : $Fuc_2GlcNAc$). To understand the biological role of xylose and fucose, synthesis of oligosaccharides lacking one or both of these residues were also needed. Disconnection to these two fragments was expected to meet this purpose.

Fragment **A** was further divided into monosaccharide (fragment **C** : Man), disaccharide (fragment **D** : GlcNAcMan), and trisaccharide (fragment **E** : ManXylGlcNAc). For the construction of β-mannoside contained fragment **E**, *p*-methoxybenzyl assisted intramolecular aglycon delivery was used as the key reaction[3]. Thus, β-mannosylation of glucosamine derivative using mannnosyl donor was performed by way of mixed acetal according to the standard protocol to afford disaccharide (ManGlcNAc) as a single stereoisomer. This disaccharide could be directly used for the glycosyl acceptor of xylosylation to synthesize fragment **E**. Then, selective desilylation under high pressure developed in our laboratry[4] was conducted to afford partially deprotected fragment **E**. It was then used as a glycosyl acceptor for subsequent glycosylation with fragment **D** to synthesize pentasaccharide ($GlcNAcMan_2XylGlcNAc$). Finally, fragment **A** was synthesized by the glcosylation between fragment **C** and penttasccharide. Fucα1-3GlcNAc, part of structure of fragment **B** was synthesized by α-fucosylation with glucosamine derivative promoted by $CuBr_2$/TBAB system[5]. Fragment **B** will also be synthesized by subsequent α-fucosylation at C-6 hydroxyl group in Fucα1-3GlcNAc.

Further assembly of these fragment toward target nonasaccharide is under investigation and will be discussed in the presentation.

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(96) **Synthesis of New Polyester from Biomass**

Kohsaku Okuyama, Daisuke Yoshida and Kenichi Hatanaka
1Institute of Industrial Science, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8050 Japan .

Biochemicals from biomass that were produced as an unavoidable by-product of wooden products or grain-basedÅ@products has attracted attention as an alternative for petrochemicals. To effectively and commercially manage resources, transformation to biochemicals will simplify processes of production. We focused an attention on 2-hydroxymethyl-2-furaldehyde (HMF) that was produced from wooden biomass by pressurized hot-water treatment processes. Reduction of HMF afforded 2,5-dihydroxymethylfurun at 92 % yield. 2,5-Dihydroxymethylfurun has two hydroxyl groups that can be utilized for polycondensation into polyesters. On the other hand, oxidation of HMF afforded 2,5-difuroic acid, which has two carboxylic acid. Partial oxidation with Ag_2O-CuO changed HMF to 5-hydroxymethyl-2-furoic acid which has one hydroxyl group and one carboxylic acid, and may undergo self-polycondensation. 2,5-Dihydroxymethylfuran, 2,5-difuronic acid and 5-hydroxymethyl-2-furonic acid were used as starting materials for the synthesis of polyesters or polyamides that are expected to be biodegradable plastics. Syntheses of polyesters were carried out by using HMF derivatives and naturally occurring carboxylic acids such as succinic acid, fumaric acid and maleic acid. Polyesters obtained from 2,5-dihydroxymethylfuran and succinyl dichloride had low molecular weight of about Mn 1000 Å` 4000. Direct polycondensation of HMF and succinic acid using N,N-dicyclohexylcarbodiimide as condensing agent gave relatively high molecular weight (Mn 6300). These polyesters from 2,5-dihydroxymethylfuran and succinic acid showed Tg (grass transition temperature) (15ÅãC) and Tm (melting point) (93ÅãC). There were no Tg and Tm for polyesters from 2,5-dihydroxymethylfuran and fumaric acid which decomposed at 208ÅãC. Polyesters from 2,5-dihydroxymethylfuran and maleic acid did not have Tg, but showed Tm (109ÅãC). Copolycondensation of 2,5-dihydroxymethylfuran with various ratios of succinic acid, fumaric acid and maleic acid were performed. The obtained copolyesters showed various thermal properties. Polycondensation using 2,5-dihydroxycarbonylfuran and self-polycondensation or polycondensation of 5-hydroxymethyl-2-furoic acid using catalysts or condensation agents are now in progress.

(97) **Synthesis of Asparagine-Linked Glycan Chains: Toward Understanding Glycoprotein Quality Control**

Ichiro Matsuo, Shinya Hagihara, Kiichiro Totani, Toshinori Kashiwagi and Yukishige Ito
The Institute of Physical and Chemical Reseach (RIKEN) and CREST Project, JST.

The endoplasmic reticulum has a quality control system for folding and maturation of newly synthesized protein. The calnexin/calreticulin-glucosyltransferase cycle plays the central role in glycoprotein quality control. Calnexin (CNX) and carleticulin (CRT) have a lectin property and recognize monoglucosylated high mannose type glycan of asparagine-linked glycoproteins as a primary ligands[1]. Thus, because of importance of the glycoprotein-CRT interaction in various biological processes, the study toward molecular levels understanding of CRT-oligosaccharide interactions has become imperative. However, The oligosaccharide binding study on CRT and CNX have been limited because of severe difficulty obtaining homogeneous sugar chains in sufficient amounts from natural sources. For that reason, we have been developing the synthetic route toward high-mannose type sugar chains, and achieved synthesis of monoglucosylated dodecasaccharide (α-$GlcMan_9GlcNAc_2$ and unnaturalβ-$GlcMan_9GlcNAc_2$). These sugar chains were subjected to interaction analysis using NMR, which revealed that carbohydrate recognition by CRT was specific to α-linked $Glc_1Man_9GlcNAc_2$[2].In this presentation, we report 1) the systematic syntheses of high mannose type sugar chains and 2) interaction analysis of synthesized sugar chains with CRT using isothermal titration calorimetry (ITC).

Systematic syntheses of high-mannose type glycan chains;

High-mannose type glycan chains ($Glc_{0\sim3}Man_9GlcNAc_2$, $Glc_{0\sim1}Man_8GlcNAc_2$ B-isomer (B) and $Glc_{0\sim1}Man_8GlcNAc_2$ C-isomer (C)) were synthesized in a convergent and stereoselective manner. The target sugar chains were divided into four oligasaccharide fragments as follows: core trisaccharide fragment including β-mannoside bond (M1GN2) [3], linear mannotriose fragment (M3), branched mannooligosaccharide fragment (M5 and M4), and glucose fragment (G1, G2, and G3). Construction of undecasaccharide fragment was achieved by condensation of M1GN2 and M3 fragment, which was followed by the coupling with M5 fragment. Selective desilylation under high-pressure conditions (1.0 GPa) gave undecasaccharide acceptor [4]. Introduction of α-glucose residue (G1) gave fully protected dodecasaccharide. Glycosylation of undecasaccharide acceptor with G2 fragment gave tridecasaccharide. Tetradecasaccharide was synthesized using G3 fragment. These oligsaccharides were eventually deprotected to give $Man_9GlcNAc_2$ (M9), $Glc_1Man_9GlcNAc_2$ (G1M9), $Glc_2Man_9GlcNAc_2$ (G2M9) and $Glc_3Man_9GlcNAc_2$ (G3M9) as propyl glycoside, respectively. Using similar strategy, G1M8(B), M8(B), G1M8(C) and M8(C) were also prepared [5].

Measurement of oligosaccharide-CRT interactions by Isothermal Titration Calorimetry;

Synthesized glycan chains (G2M9, G1M9, M9, G1M8(B), and G1M8(C)) were subjected to binding studies with recombinant CRT using isothermal titration calorimetry. G1M9, G1M8(B) and G1M8(C) had detectable affinities toward CRT ($Ka \times 10^{-6}M^{-1}$ = 5.3, 3.5, and 4.4, respectively). On the other hand, binding of di-glucosylated (G2M9) and nonglucosylated (M9) glycan chains were not detectable. It was revealed that α-conjugated monoglucose residue is critical for CRT binding and structure of branched mannooligosacchride linked to *C*-6 of β-mannose residue affects the binding affinity.

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(98) Di-*tert*-butylsilylene(DTBS) Group-Directed α-selective Galactosylation: Effect of Leaving Groups of Glycosyl Donor.

Akiyoshi Kimura[1], Akihiro Imamura[1,2], Hideharu Ishida[1] and Makoto Kiso[1,2]
[1] Department of Applied Bioorganic Chemistry, Gifu University, 1-1 yanagido,Gifu 501-1193, Japan, [2] CREST, Japan Science and Technology Agency (JST), 1-1 Yanagido, Gifu 501-1193, Japan.

The feasibility of oligosaccharide synthesis, in general, mainly relies on the stereoselectivity in the glycoside formation where the outcome of the α- and β-glycoside would be produced simultaneously. In the situation of *galacto*-type glycoside formation incorporated within oligosaccharide synthesis, β(1,2-*trans*)-selectivity can be efficiently accomplished by the neighboring effect of various acyl functionalities mounted on the C-2 amino group of the glycosyl donor, by nitrile solvent effect under thermodynamically controlled condition, or by tethering of the glycosyl donor to the glycosyl acceptor. For α(1,2-*cis*)-selective glycosylation, on the contrary, the glycosyl donors with non-neighboring functionality on C-2 have been commonly exploited in order to maximize the anomeric effect, often with the aid of ethereal solvent effect. Recently, we have discovered[1] an unusual α-galactosylation using phenylthioglycoside of 4,6-*O*-di-*tert*-butylsilylene(DTBS)-protected galactose derivatives as a glycosyl donor, which was not hampered by the neighboring participation of C-2 acyl functionality such as NTroc and OBz. The power of the DTBS effect has been exemplified by the coupling reaction with various glycosyl acceptors.
As part of our study to extend the generality of this methodology, we describe herein the effect of leaving groups of the glycosyl donors. Based on the structure of the original galactosamianl donor, phenyl 2-deoxy-4,6-*O*-di-*tert*tert-butylsilylene-3-*O*-(2,2,2-trichloroethoxycarbonylamino)-1-thio-β-D-galactopyranoside, we designed and prepared a series of novel galactosaminyl donors in which phenylthio group was replaced with fluoro, bromo, trichloroacetimidate and methylthio groups. The donors prepared were coupled with 2-adamantanol as a common glycosyl acceptor under the conditions which are optimized for each leaving group. Coupling of the methylthio donor in the iodonium-promoted condition resulted in a high α-selectivity. Similary, the fluoride and the trichloroacetimidate also showed high α-selectivity, respectively. This result strongly inspired us to apply this methodology to orthogonal synthetic strategy. On the other hand, the bromide gave the β-glycoside when coupled in the presence of insoluble silver silicate as a promoter.
Finally we tried the coupling of the phenylthio derivative with a suitably protected serine derivative as an application of this methodology to natural compounds, and succeeded in the É—-selective synthesis of the desired α-galactosaminyl serine which, is the key structure of mucin-type glycoprotein.
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(99) Characterization and Utilization of a β-primeverosidase-like *endo*-β-glycosidase from *Penicillium multicolor*

Satoshi Amarume[2], Takeomi Murata[1], Toshitaka Tsuruhami[2], Shigeharu Mori[2], Jun Hiratake[3], Kanzo Sakata[3] and Taichi Usui[1]
[1] Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan, [2] Amano Enzyme Inc. Gifu R&D Center, 4-179-35 Sue-cho Kakamigahara, Gifu 509-0108, Japan, [3] Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan.

A β-primeverosidase has been purified from the crude enzyme extract prepared from fresh leaves of Yabukita (*Camellia sinensis* var. *sinensis*) for Japanese green tea, Shuixian (*C. sinensis* var. *sinensis*) for oolong tea, and a cultivar (*C. sinensis* var. *assamica*) for black tea. However, the physiological role of the disaccharide that released by the hydrolysis of aroma precursor has not been elucidated. From such an aspect, a large amount of primeverosides as aroma precursors will be needed to study the function. We have recently reported that a β-primeveroside-hydrolyzing enzyme from *Aspergillus fumigatus* AP-20 cleaved in an *endo*-manner *p*-nitrophenyl β-primeveroside (6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside) into primeverose and *p*-nitrophenol. However, it is not always available on the utilization, because of pathogen. For this purpose, we further searched for β-primeverosidase-like enzyme from other microbial sources, because the finding of such a kind of enzyme will expand to the possible application in biotechnology, for example, the control of aroma function or high recovery of aroma extraction, and the enzymatic synthesis of β-primeveroside as aroma precursor. In present results, a β-primeverosidase-like *endo*-β-glycosidase from the culture filtrates of *Penicillium multicolor* was purified to apparent homogeneity by precipitation with ammonium sulfate followed by successive chromatographies on Phenyl Sepharose, β-galactosylamidine affinity columns etc. The molecular mass was estimated to be 50 kDa by SDS-PAGE and the isoelectric point to be pH 4.5 by isoelectric focusing. The purified enzyme was highly specific for *p*NP β-primeveroside, which was cleaved in an *endo*-manner into a primeverose product and a *p*-nitrophenol product. The unique *endo* β-glycosidase also hydrolyzed naturally occurring aroma precursors of eugenyl, benzyl, 2-phenylethyl, (*Z*)-3-hexenyl and geranyl β-primeverosides to liberate the corresponding aroma compounds, even though at the low rate. From hydrolytic action and kinetics studies, we suppose a subsite structure so that *p*NP β-primeveroside has a matching shape, which can accommodate chain of three residues to fit into the active site. Furthermore, the present enzyme was shown to be useful as a synthesis tool for obtaining eugenyl, benzyl, 2-phenylethyl, (*Z*)-3-hexenyl and geranyl β-primeverosides, which were effectively synthesized by β-primeverosyl transfer reaction from *p*-nitrophenyl β-primeveroside to various aroma compounds in aqueous-aroma two-phase system.

(100) Enzymatic Synthesis of Chondroitin by Chondroitin Polymerase from *Escherichia coli* strain K4

Nobuo Sugiura[1,2], Hideo Mochizuki[1,2], Hiroshi Maeda[2] and Koji Kimata[1]
[1] Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-1195, Japan, [2] Central Research Laboratories, Seikagaku Corporation, Tateno, Higashiyamato, Tokyo 207-0021, Japan.

Chondroitn sulfate (CS) is a glycosaminoglycan having a repeating disacharide unit of [β4-D-glucuronic acid (GlcUA) -β3-N-acetyl-D-galactosamine (GalNAc)] with sulfate residues at various positions. Proteoglycans carrying CS chains are localized in the extracellular matrix and on cell surfaces of various tissues. The biosynthesis of CS proteoglycans occurs in the Golgi apparatus. First, the linkage tetrasaccharide, GlcUA-Gal-Gal-Xyl, is synthesized onto a serine residue of the core protein by sequential addition. Then a chondroitin chain is elongated on to the linkage by the alternating addition of monosaccharide units of GalNAc and GlcUA. During the polymerization, the chain undergoes sulfation at various positions with a variety of sulfotransferases. Therefore chondroitin, intermediate for the biosynthesis of CS, may serve as a useful tool for elucidation of the mechanisms of CS biosynthesis. Many animal species produce CS proteoglycans, including vertebrates, insects, mollusks, and nematodes. Although bacteria do not contain CS proteoglycans, *Escherichia coli* (*E. coli*) strain K4 produces a polysaccharide similar to chondoritin in the capsule (K4 antigen) that has fructose branched at position C-3 of the GlcUA residue. Polymerization of K antigen occurs at the inner face of the cytoplasmic membrane with phospholipid conjugated 2-keto-3-deoxyoctulosonic acid (KDO) at the reducing terminal, and the polysaccharide is translocated across the outer membrane and then forms the extracellular layer of the capsule. We identified a gene encoding a bifunctional glycosyltransferase from *E. coli* strain K4. The enzyme, designated chondroitin polymerase of strain K4 (K4CP), contained two glycosyltransferase active sites; GalNAc transferase site at the N-terminal side and GlcUA transferase site at the C-terminal side. K4CP transferred GalNAc and GlcA residues alternately to chondroitin oligosaccharide acceptor substrate with UDP-GalNAc and UDP-GlcUA as donor substrates. The recombinant soluble enzyme elongated chondroitin polysaccharide chain up to 20 kDa (approximately 100 sugars). Point mutations of [D241K] and [D521K], at each of the UDP-sugar binding motif (DXD) in the two glycosyltransferse active sites into DXK, abrogated the respective transferase activity. Interestingly, mixture of the point mutant enzymes recovered the polymerase activity. Å@Matrix-assisted laser desorption ionization and time-of-flight mass spectrometry (MALDI-TOF-MS) has recently been used to determine the structure of glycosaminoglycan chains. We identified the products of chondroitin oligo- and poly-saccharides synthesized by K4CP and the two mutant enzymes up

to approximately eicosasaccharide in detail by analysis of MALDI-TOF-MS. The analysis was operated in negative ion mode using 2,5-dihydroxy benzoic acid (DHB) as matrix. This may be useful for elucidating the mechanism of complex polysaccharide synthesis.

(101) **Characteristics of an α 2,6-sialyltransferase produced by a marine bacterium, *Photobacterium damsela* JT0160**

Takeshi Yamamoto, Yoshimitsu Takakura and Hiroshi Tsukamoto
Plant Innovation Center, Japan Tobacco Inc., Higashibara, Iwata, Shizuoka 438-0802, Japan.

An abundant supply of sialyloligosaccharide is necessary for analyzing the function of sialyloligosaccharides. There are two major methods of sialylation, chemical sialylation and enzymatic sialylation. Usually, chemical sialylation is furnished in low yield with low stereoselectivity. On the other hand, enzymatic sialylation takes advantages in those problems. Thus, the sialyltransferase is one of the most demanded enzymes. In general, bacterial enzymes are more stable and productive compared to mammalian enzymes. In the course of the screening for bacteria, we identified a marine bacterium, *Photobacterium damsela* JT0160, that produces a sialyltransferase and we purified a β galactoside α 2,6-sialyltransferase from this bacterium. We previously reported that the enzyme had unique characteristics. The α 2,6-sialyltransferase had a unique acceptor specificity compared with that of mammalian sialyltransferases. For example, the enzyme transferred NeuAc from CMP-NeuAc to 3'-sialyllactose and 2'-fucosyllacotose with high efficiency respectively. This enzyme also transferred NeuAc through α 2,6-linkage onto N-acetyl-galactosamine. Therefore, it was found that the enzyme could act as a N-acetylgalactosaminide α 2,6-sialyltransferase. In point of the productivity of the sialyltransferase, the maximum productivity of this enzyme in *P. damsela* JT0160 reached 550 U/L culture. The gene encoding the enzyme has no homologous regions to the cloned genes of mammalian and other bacterial sialyltransferases, indicating that the primary structure of the enzyme is quite different from those of known sialyltransferases. Recently, we found that the sialyltransferase activity of *P. damsela* JT0160 was increased in the presence of sodium chloride in both cases of using crude and fully purified enzyme, but such a phenomenon was not observed when divalent cation existed in an assay mixture. In this presentation, we will be focusing on the effect of sodium chloride on enzyme activity and enzymatic syntheses of sialyloligosaccharides. We also show some other studies about this enzyme.

(102) **Facile Formation of a Macrocyclic Structure of Tricolorin A Using Intramolecular Glycosidation**

Kazuteru Kaneko, Natsuko Yanagiya, Tetsuya Furuike and Nobuo Sakairi
Graduate School of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan.

Convolvulaceae family such as *Ipomoea stans*, *I. orzabensis*, *I. operculata*, and *I. leptophylla* have an extensive history of use in Mexican folk medicine and traditional agriculture. *I. tricolor* Cav., which has been cultivated at the fallow period of a sugarcane field, has been used for many years to prevent weed growing. A macrocyclic hetero-oligosaccharide, named Tricolorin A, was isolated as a crucial substance of this bioactivity. This compound has been reported to work not only phytogrowth inhibitor but also cytotoxin against human breast cancer cells. It is quite interesting to note that the biological activities were lost when its cyclic structure was decomposed. From a viewpoint of its total synthesis, the formation of this macrolactone structure has been one of the greatest obstacles. Although such successful methods as intramolecular esterification or ring closing metathesis have been reported, these reactions are needed to perform under high dilution conditions. Here, we report an alternative method for the construction of the macrolactone structure using intramolecular glycosidation. The macrolactone residue of Tricolorin A involves D-fucose-β-(1, 2)-D-glucose- disaccharide unit. We were to introduce the two monosaccharides to a hydroxyl carboxylic acid and then cyclize by intramolecular glycosidation. In this study, we have used lauryl thioglycosides, which were prepared from dodecanthiol free of stink. At first, we attempted to synthesize an intermolecular esterification between D-glucoside and lauryl thiofucoside having unprotected hydroxyl group at 2- and 3-positions, respectively. Although the yield of the desired substrate for intramolecular glycosidation was low due to unselective esterification and migration of a protecting group, activation of the laurylthio group by methyl trifluoromethanesulfonate gave a macrocyclic compound in 65% yield. Next, We examined protection of unnecessary hydroxyl group before the esterification. Good results were obtained when we used 11(S)-(2-O-chloroacetyl-3,4-O-isopropylidene-β-D-fucopyranosyloxyl)-hexadecanoic acid and laurly 2-O-levulinyl-4,6-O-benzylidene-1-thio-β-D-glucopyranoside as the substrates for the condensation with dicyclohexylcarbodiimide in dichloromethane. Further investigations to optimize the conditions of this intermolecular esterification and the results of the intramolecular glycosidation will be discussed. In addition, we attempt to synthesize the macrolactone of Tricolorin A by using different monosaccharides and hydroxyl carboxylic acids in order to investigate the effect of constituents toward macrolactonization. Base on native Tricolorin A is composed a D-glucose, D-fucose, two L-rhamnoses, and a jalapinolic acid (11(S)-hydroxyhexadecanoic acid) as the aglycon, we are interested to substitute the D-galactoside for a D-glucoside, the lauryl thioglucoside for a lauryl thiofucoside, and the 11-hydroxyundecanoic acid for a 11(S)-hydroxyhexadecanoic acid and so on. The synthetic routes and results will be discussed.

(103) **Protein Modification by C-Glycosylation: Synthesis of C-Mannosyl Tryptophan and Its Relative Peptides**

Shino Manabe[1,2] and Yukishige Ito[1,2]
[1] RIKEN, Hirosawa, Wako-shi, Saitama, 351-0198, Japan, [2] CREST, JST, Honcho, Kawaguchi, Saitama, 332-0012, Japan.

Glycosylation is one of the most important post- or co-translational modifications of proteins, which affects the biological activities of the parent proteins by influencing the higher-order structure. This modification is classified into two subtypes, namely N-linked type and O-linked type. Recently, a highly novel variant of glycoproteins that incorporate a C-glycosylated amino acid were identified in various proteins such as IL and thrombospondin. The total synthesis of C-glycosyl amino acid, namely, C-mannosylpyranosyl-L-tryptophan and related peptides were successfully achieved. The mannose and tryptophan moieties were connected via ring opening of benzyl-protected 1,2-anhydro-mannose by a lithiated indole derivative. After the functional group conversion and deprotection steps, the glyco-amino acid was synthesized in a concise and stereoselective manner, in high overall yields. Furthermore, intermediate azide acid can serve as a useful building block for peptide elongation. The stereoisomer, glucosylpyranosyl-L-tryptophan was synthesized in a similar way.

(104) **Synthesis of Novel Macromolecular Compounds Containing Cyclodextrin**

Kiyoshi Tamura, Naoko Yoshie and Kenichi Hatanaka
Institution of Industrial Science, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan.

Gel is the 3-dimensionally crosslinked polymer. The structure of gel is categorized by the type of crosslinks. In physical gel, polymer chains are crosslinked by intermolecular interaction, while in chemical gel, polymer chains are crosslinked by covalent bonds. Two kinds of gels are the same in the point that polymer chains are fixed.

We are researching on novel gel made from cyclodextrin(CD) and polymer. In this gel, crosslinks can be formed neither by covalent bond nor intermolecular interaction. CD is the cyclic compound and can include various polymers in its hydrophobic cavity. Therefore, polycondensation of a difunctionarized polymer with dimodified CD can give a gel in which some CD is penetrated by polymer chain to form sliding crosslinks. It will be expected that this gel will be very flexible and have high strength because CD can move along the polymer chains and CD and polymer chains are linked by covalent bonds. Moreover, if the included linear polymer component is biodegradable, the whole gel must be biodegradable and can be applied to biomaterials because some enzymes can degrade CD, which is another component.

In this study, diaminoCD, which has two amino groups at upper rim of g-CD, was synthesized as dimodified CD and DC-PEG, which has two carboxyl groups at the both ends of polyethylene glycol, was used. The gel was synthesized by polycondensation of diaminoCD and DC-PEG (Mw = 2925) in dimethyl sulfoxide (DMSO). The gelation was finished in 5 minutes. DMSO is not essential for the gelation because the synthesized gel was not collapsed in plenty of water after a few days. The synthesized gel is biodegradable because the synthesized gel was collapsed by a-amylase. Moreover, after 1 month, the synthesized gel was collapsed in water solution. These results imply that the gel made from diaminoCD and DC-PEG can be applied to biomaterial for example implant material, because the synthesized gel was collapsed not in short period but in middle range period. When using unmodified g-CD instead of diaminoCD, the gelation did not occurred. This result shows that amino groups of diaminoCD or

amide bonds that are generated from diamino CD and DC-PEG attribute the formation of the gel.
When saturated g-CD aqueous solution was added to DC-PEG, white solid was precipitated. ^{1}H-NMR spectrum of this precipitate in DMSO showed broadening of the peak of H-3 and H-5 of g-CD, and upfield shift of the peaks of methylene protons that is next to carboxyl groups of DC-PEG. These broadening and upfield shift of peaks indicate that the formation of the inclusion complex of g-CD and DC-PEG, which implies that the oxyethylene chains of DC-PEG can penetrate the cavity of diaminoCD in DMSO solution. Therefore, some CD molecules can be penetrated by DC-PEG chain in the synthesized gel.

(105) **Production and Immobilization of Human Glycosyltransferases at the Yeast Cell Surface Through Fusion with Cell Wall Pir Proteins**.

Yoh-ichi Shimma, Fumie Saitou, Fumi Oosawa and Yoshifumi Jigami
Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology
AIST central 6, Tsukuba, Ibaraki, 305-8566, Japan.

Taking an advantage of the collection of nearly two hundred human glycosyltransferase genes in AIST, we have tried to develop a production system of various human glycosyltransferases required for the enzymatic synthesis of various type oligosaccharides. Because enzymatic reactions are useful to remodel oligosaccharide structures of glycoproteins, glycolipids, and living cells, it will be required to produce many kinds of glycosyltransferases in a large scale. To this end, truncated glycosyltransferase genes that eliminate their N-terminal transmembrane regions were constructed and introduced into the Gateway entry vector, which is convenient to introduce cloned genes into various expression vectors. We planed to express fifty human glycosyltransferase genes, which are well characterized and important for synthesizing useful oligosaccharides, in yeast Saccharomyces cerevisiae. The yeast cell wall mainly consists of β 1,3-glucan, β1,6-glucan, chitin, and mannoproteins. Among a kind of mannoproteins, Pir (protein with internal repeats) proteins are known to bind to the β1,3-glucan, a major component of the yeast cell wall, in an alkaline sensitive manner. Pir proteins form a Pir1~4 family with different numbers of above internal repeats and the genes for these family show significant differences in their expression patterns, although little is known for their physiological functions. Since endogenous glycosyltransferases generally contain one transmembrane region near the N-terminus to reside at the Golgi membrane, we have fused Pir proteins with the glycosyltransferases that are truncated for their N-terminus transmembrane regions to express them at the yeast cell wall after remaining their enzyme activities. We have measured their enzyme activities by using the yeast cell wall as an enzyme source. The preparation of cell wall fractions is very simple, which includes a homogenization of intact yeast cells with glass beads and a precipitation of cell wall after centrifugation of cell extracts. No protein purification and immobilization process were necessary due to the display of immobilized enzymes at the cell surface. So far, we expressed more than twenty genes and detected fourteen enzyme activities among them, which include FUT1, FUT3, FUT6, FUT8, ST3Gal I, ST3Gal IV, ST6Gal I, and ppGalNAcT.

(106) **Synthetic Study on HPG Series Gangliosides, New Species Isolated from the Sea Cucumber; Construction of Fucosyl α(1→4)/α(1→8) Sialic Acid**

Yukari Katano[1], Hiroyo Shimizu[1], Yusuke Koike[1], Sachiko Koizumi[1], Hiromune Ando[2], Hideharu Ishida[1] and Makoto Kiso[1]
[1] Department of Applied Bioorganic Chemistry, Gifu University,1-1 Yanagido, Gifu, Gifu, Japan 501-1193,
[2] Life Science Research Center, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan.

As more and more biological functions of gangliosides are being revealed, their facile, stereocontrolled synthesis is strongly reguried. We have succeeded in the total synthesis of a variety of gangliosides including their analogs and derivelities, and have contributed to the elucidation of their biological functions at the molecular level. Recently, new species of gangliosides, namely HPG-series gangliosides, have been isolated from the sea cucumber. In this paper, we describe the synthetic study on HPG-1[1)] and HPG-7[2)] gangliosides as part of an effort to develop the novel medicinal resourses from marine products.
One important point in the synthesis of the entitled gangliosides is the construction of fucosyl α(1→4)/α(1→8) sialic acid structures, which were discovered as the substructures of gangliosides for the first time, and have never been chemically synthesized. Because our previous results in the condensation of C-4/ C-8 hydroxyls of Neu5Ac unit 2-phenylthio Neu5Ac donor suggested that the C-4/ C-8 hydroxyls are less reactive than anticipated, we developed novel sialic acid derivative '1,5-lactam-sialic acid' as a glycosyl acceptor. The hydroxyls at C-4/ C-8 are far from the hamper by C-5 amido moiety in this structure. Starting from the introduction of phenylthio group into Neu5TFAc[3)], the featuring 1,5-lactam-sialic acid was efficiently synthesized *via* one-pot deblocking N-TFAc and lactamization in methanolic sodium methoxide. The regioselective 8,9-O-isopropylidenation of this key intermediate gave a glycosyl acceptor suitable for fucosyl α(1→4) sialic acid. A glycosyl acceptor for fucosyl α(1→8) sialic acid was prepared by the 8,9-O -benzylidenation, 4,7-O -acetylation and regioselective ring opening of benzylidene acetal of the key intermediate. Coupling reactions of the fluoride donor of fucose4) and 1,5-lactam acceptors obtained above gave the desired fucosyl α(1→4) sialic acid in 55% (α:β=7:1) and fucosyl α(1→8) sialic acid in 86% (α:β=3:1), respectively.
The disaccharide synthesized are now employed for the assembly of HPG-1 and HPG-7 gangliosides.

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(107) **Facile Synthesis of the 3-*O*-alkyl-D-glucose Derivatives and their Antifungal and Fruiting-inducing Activities Against a Basidiomycete**

Takeshi Nishimura, Yumi Magae and Seiji Ohara
Department of Wood Improvement, Forestry and Forest Products Research Institute, PO Box 16,
Tsukuba Norin Kenkyu Danchi-nai, Ibaraki 305-8687, Japan.

INTRODUCTION: Fruit body differentiation is an important process for mushroom cultivation. Environmental factors like light, temperature and nutrition depletion are known to be effective on the fruit body differentiation. Previously, we reported the fruit body inducing activity of sucrose ester of fatty acids (SE) and triterpenoid glycoside (saponin) on *Pleurotus ostreatus*[1)]. Furthermore, simple glucose derivatives that exert surfactancy; i.e. 3-*O*-octyl-D-glucose and 3-*O*-decyl-D-glucose showed fruit body inducing activity on *P. ostreatus*[2)]. From our previous work using various surfactants with or without a carbohydrate moiety, we concluded the following: 1) the proper hydrophilic-lipophilic balance was necessary for fruiting-inducing activity; 2) the sugar-containing structure such as 3-*O*-alkyl-D-glucose derivatives, which can not be metabolized by *P. ostreatus*, was also necessary for the activity. In this presentation, we describe the antifungal and the fruiting-inducing activities of 3-*O*-alkyl-D-glucose derivatives against *Fomitopsis palustris* FFPRI 0507, an important brown rot fungus designated in Japanese industrial standard. Facile synthetic routes toward the series of 3-*O*-alkyl-D-glucose derivatives (alkyl chain length; n = 8-18) with different hydrophilic-lipophilic balance were established to investigate the biological activities and the structure-activity relationships.

RESULTS AND DISCUSSION: Series of 3-*O*-alkyl-D-glucose derivatives were successfully synthesized from 1,2,5,6-diisopropylidene-D-glucose according to the modified method of Ikekawa *et al.* but with a greatly improved overall yields (about 70%)[3)]. Their structures were confirmed by FAB-MS and ^{1}H- and ^{13}C-NMR spectrometry. *F. palustris* was inoculated on the basal malt extract agar (MA; 20 ml of 2% malt extract,1.8% agar) in Petri dishes (9 cm in diameter) containing 0.05% of the synthetic samples. The appearance of primordia (fruiting initials) and fruit bodies were observed on the medium between 10 and 20 days after incubating at 17 ˚C under 100 lux illumination. Also each sample showed more or less a growth inhibitory effect on the *F. palustris* mycelium. Unexpectedly, the inhibitory effects did not always increase with the numbers of alkyl chain carbons but the odd-even like effect was observed.Further investigations on how mushrooms discriminate the chemical structures of 3-*O*-alkyl-D-glucose derivatives are now in progress.

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(108) **Facile Procedure to Introduce ω-mercaptoalkyl Residue into Glucose and Sialyl α (2→3)lactose as a Versatile Spacer for Further Conjugations**

Yuki Kato[1], Mikio Ishihara[1,2], Hideharu Ishida[1] and Makoto Kiso[1]
[1] Department of Applied Bioorganic Chemistry, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan, [2] Tokyo Kasei Kogyo, 4-10-2 Nihonbashihonmachi Tyuouku Tokyo 103-0023, Japan.

The development of efficient routes for the preparation of complex oligosaccharide or carbohydrate conjugates has contributed to the successful progress of glycobiology. Synthesis of the conjugate of synthetic carbohydrate antigens and carrier proteins is one of the successful achievements in this area, and which is now being further investigated to be applied for vaccination of tumors. One of the important points to synthesize such conjugates is the development of suitable linkers and spacers. Danishefsky *et al.* developed MBS reagent which couple thiol and amino function in a highly efficient manner(1). Because amino function naturally occurs in proteins, the only problem is how to establish a methodology of introducing thiol function into oligosaccharide structure. In this paper, we describe a facile procedure to couple ω-melcaptoalkyl residue to oligosaccharides.

We employed glucose and sialyl α (2→3)lactose as model (oligo)succharides. In order to assess the effect of chain length on chemical reactivity as well as biological efficiency, we selected 2-mercaptoethanol and 6-mercaptohexanol as spacer arms. Commercially available 2-mercaptoethanol and 6-mercaptohexanol were treated with methoxycarbonylsulfenyl chloride to give the intermediate, which were then converted into the t-butylsulfenylated derivatives(2)(3) as the desired acceptors by the treatment with 2-methyl-2-propanethiol in almost quantite yields. Coupling of the trichloroacetimidate of perbenzoylated glucose with the suitable protected mercaptoalcohol acceptors gave the desired β-glycosides in 82% and 75% yields, respectively. Finally, deprotection of benzoyl group by Zemplen condition followed by the treatment with TCEP·HCl afforded the fully deprotected compounds in 98% and 86% yields. Similar coupling of sialyl α(2→3)lactose donor with the 6-mercaptohexanol derivative as an acceptor followed by the complete deprotection gave the gangliosde GM3-related oligosaccharide probe. The compounds obtained are now under the investigation for the coupling with carrier proteins.

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(109) **Synthesis of the Dibranched Core OS expressed in Neisserial LOS Using a Versatile Hep Intermediate**

Kazuyuki Ishii, Kiyoka Toyota, Tsuyoshi Ichiyanagi and Ryohei Yamasaki
Department of Biochemistry and Biotechnology, Tottori University, Tottori, Japan.

Pathogenic gram-negative bacteria such as Neisserial and Haemophilus species produce glycolipid antigens called lipooligosaccharide (LOS). LOS consists of the oligosaccharide (OS) and the lipid A, and the OS has been considered as vaccine targets against those pathogenic bacteria. The OS expressed in LOS produced by the above bacteria has branched structures; the 2,3-, 3,4-, or 2,3,4 positions of a common carbohydrate Heptose are glycosylated with a monosacchride or smaller oligosaccharide (Hep). For example, a strain 15253 of Neisseria gonorrhoeae produces LOS containing the 2,3- and 3,4-dibranched Hep. These dibranched structures are also expressed in LOS and LPS of different spieces of gram-negative bacteria. For the synthesis of such branched OS structures, suitable protected Hep derivatives are essential to build up specific structures, and sometimes the use of a versatile Hep derivative could be critical to accomplish the synthesis. Frequently used Hep derivatives for the synthesis are As a versatile Hep derivative for the synthesis of the branched Hep structures, we examined the use of a silyl ether derivative. Selective protection of the Hep 3-OH has been achieved by selective *O*-alkylation of a 2,3-diol using either a phase-transfer method or a tin intermediate. The drawback of the *O*-alkylation method is that the reaction is not highly regioselective and tends to give the 3-*O*-alkyl ether only in moderate yields. Alternatively, the 3-OH acceptor has been prepared by esterification of a 2,3-orthoester and subsequent treatment with aqueous TFA. We found that silylation of a Hep3,4-diol derivative regioselectively proceeds to the corresponding 3-*O*-silyl ether in high yields. By using this silyl ether, we constructed 2,3 and 3,4-di-branched structures. However, the yield of chloroacetylationÅ@of the 2,3-orthoester is not as high as that of acetylation, which may be a drawback for the synthesis of the 3,4-di-*O*-substituted Hep. In addition, the 2,3-orthoester intermediate may not be directly utilized for the synthesis of the 2,3-dibranched Hep structure. In contrast, both regioselective 3-*O*-silylation and regeneration of the 3-OH were accomplished in high yields, and the 3-*O*-silyl ethers 4 and 6 can be directly employed for the synthesis of the 3,4-di-*O*-substituted Hep. Also, by using an appropriate silyl ether stable under hydrogenolysis conditions, the 2,3,4-tri-*O*-substituted Hep expressed in Neisserial and Campylobacter LOS can be synthesized from the 3-*O*-silyl intermediate. Therefore, in terms of the ease and yields of protection and deprotection, and the versatility of the 3-*O*-silyl ether intermediate for the synthesis of branched Hep structures, regioselective 3-*O*-silylation of the 2-*O*-substituted Hep has advantages over the existing procedures described above. Although we did not examine silylation of a 2-*O*-substituted mannoside in the present study, its regioselective 3-*O*-silylation could be accomplished in the similar manner, and this *O*-3 silylation would be also useful for the manipulation of the secondary hydroxyl groups of mannose. In summary, we found that the 3,4-diol derivatives of both the 2-*O*-Bn Hep and a-GlcN_3-(1-2)-Hep were regioselectively silylated to give the corresponding 3-*O*-protected products in high yields. By coupling the 3-OH acceptor generated from the 3-*O*-silylated a-GlcN_3-(1-2)-Hep with the per-*O*-benzylated lactosyl trichloroacetimidate, we synthesized the 2,3-dibranched Hep structure, a-Lac-(1-3)-[a-GlcN_3-(1-2)]-Hep in a high yield and converted the azide to the acetamide by hydrogenation and subsequent *N*-acetylation. Thus, we constructed the 2,3-branched Hep, a-Lac-(1-3)-[a-GlcNAc-(1-2)]-Hep present in 15253 LOS. The 3-*O*-silyl derivative of the 2-*O*-substituted Hep is a useful intermediate for the 2,3-dibranched and could be utilized for the synthesis of both the 3,4-dibranched and the 2,3,4-tribranched structures.

(110) **Design and Synthesis of Bacterial Ceramidase Inhibitors**

Masamitsu Miyamori[1], Masanori Yamaguchi[1], Hideharu Ishida[1], Aki Ogura[2], Hatsumi Mondyusyo[2], Makoto Ito[2] and Makoto Kiso[1]
[1] Department of Applied Bioorganic Chemistry, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan, [2] Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan.

Ceramide (Cer; *N*-acylsphingosine) has been discovered to be a potent lipid mediator of many cellular proliferation functions, including differentiation and apoptsis. Moreover, Cer functions as a water retainer as well as a permeability barrier by forming a multi-lamellae structure in the stratum corneum of the mammalian epidermis.

In lesions of atopic skin, Cer content was found to have decreased. This symptom has been thought to be significantly related to some etiological aspect of atopic dermatitis. A change in epidermal permeability caused by a decrease of Cer would permit the invasion of allergens or irritants, which is associated with the pathogenesis of atopic dermatitis. Our recent findings[1)] have suggested that bacterial ceramidase (CDase) may cause the deficiency of Cer in the stratum corneum of atopic skin, since the lesions of atopic skin were later infected with CDase producing bacteria. We began the study on the design and the synthesis of Cer analogs which are expected to be CDase inhibitors[2)], as it has become necessary to find inhibitors of bacterial CDase from a clinical point of view. In this paper , we describe the synthesis and CDase inhibitory activity of novel classes of Cer analogs.

Initially, we designed a couple of Cer analogs in which the amide linkage was replaced with the ester linkage. The desired compound was synthesized by employing the key intermediate, (2R,3R,4E)-1,3-O-benzylidene-4-eicosene-1,2,3-triol[3)]. Another class of analogs were designed as derivatives of diacyl glycerol, in which oleic acid and myristic acid were introduced to the glycerol skeleton by an ester linkage to give six kinds of the desired compounds. The effects of compounds on CDase activity has been examined by the procedure reported by N. Okino *et al.*[4)]. Among these compounds, the diacyl glycerol derivatives were found to be effective in inhibiting the activity of *Pseudomonas* CDase. The sphingoid-based derivatives had very little inhibitory activity on bacterial CDases.

In summary, diacyl glycerol derivatives appear to be a potential compound for clinical use as inhibitors of bacterial CDases.

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(111) **Highly α-specific 2-*O*-acylation of KDO Using Alkyl and Aryl Isocyanates**

Tsuyoshi Ichiyanagi and Ryohei Yamasaki
Department of Biochemistry and Biotechnology, Tottori University, Tottori, Japan.

Lipooligosaccharide (LOS) produced by gram-negative bacteria consists of the oligosaccharide (OS) and lipid A, and the core OS expressed in LOS is considered to be vaccine targets against pathogenic mucosal bacteria. Since the reducing end of the intact OS, 3-deoxy-D-*manno*-oct-2-ulosonic acid (KDO) is α-linked to the lipid A, connecting the linker between the synthesized OS-KDO and protein via the intact linkage could be essential for the development of effective conjugate vaccines. In order to develop a convenient conjugation method, we have chosen to use KDO as a nucleophile and halogen-containing alkyl/ aryl isocyanate as an electrophilic linker that could be directly linked to protein after the coupling with KDO. Reaction of 4,5,7,8-*tetra*-*O*-acetyl-3-deoxy-α-D-*manno*-oct-2-ulosonate (1) with 2-chloroethyl isocyanate in toluene gave two products. By 2D NMR (DQF-COSY, HMQC, HMBC, and ^{1}H coupled QUAT) and ESI-MS analyses, the major and minor products were found to be have an a-spiroketal structure (81-83%) and a ketal of dimeric isocyanate adduct (11-13%), respectively. The combined yields (94%) of the two products demonstrating that this reaction is highly α-specific to give the KDO of the desired anomeric configuration in high yields. Next, we also examined the reaction of 1 with an aryl derivative to extend the utility of isocyanate as an electrophile, and the products obtained were identified in a similar manner as described earlier. Similar treatment of 1 with 4-(chloromethyl)phenyl isocyanate gave the corresponding α-spiroketal (31-40 %) together with numerous side products. However, the use of THF as a solvent suppressed side reactions to give the spiroketal as a sole product in 81% yield, which also showed that the *O*-2 acylation takes place with the aryl isocyanate in a same stereoselective manner. Thus, we demonstrated that anomeric acylation of I with the alkyl and aryl isocyanates using as electrophiles are stereospecific to yield the α-spiroketal products in high yields. The present anomeric acylation could be developed to a convenient method to conjugate the OS via the intact linkage to protein.

(112) **In vitro O-glycosylation of E. coli-produced Therapeutic Proteins Using Recombinant Glycosyltransferases**

Shawn Defrees[1], Zhi-Guang Wang[1], Arthur E. Scott[1], Jin Wang[1], Ruye Xing[1], David Zopf[1], Dominique L. Gouty[1], Eric R. Sjoberg[1], Krishnasamy Panneerselvam[1], Els C.M. Brinkman-Van der Linden[1], Robert J. Bayer[1], Mads A. Tarp[2] and Henrik Clausen[2]
[1] Neose Technologies, Inc. Horsham, PA and San Diego, CA, [2] Faculty of Health Sciences, School of Dentistry, Copenhagen, Denmark.

Non-glycosylated therapeutic proteins expressed in E. coli can be made into glycoproteins by the selective introduction of GalNAc at specific serine and threonine residues by in vitro treatment with a recombinant polypeptide O-GalNAc-transferase.† We have demonstrated this with three therapeutic peptides currently in clinical use which have endogenous O-glycosylation sites: G-CSF, INF-α2b, and GM-CSF. Since there is no consensus amino† acid sequon for O-glycosylation, members of a family of recombinant polypeptide O-GalNAc transferases were screened for activity on synthetic peptides covering natural O-glycosylation sites.† A single enzyme capable of introducing GalNAc onto the natural site on peptides from all three proteins also was shown to O-glycosylate the natural site on all three intact proteins which had been expressed in E. coli.† These neoglycoproteins may subsequently serve as acceptor substrates for other glycosyltransferases in vitro to create mammalian-type O-linked glycans.

(113) **Chemoenzymatic Synthesis of CMP-sialic Acid Analogs and Sialates Using Microbial Enzymes**

Xi Chen, Hai Yu, Hui Yu and Rebekah Karpel
Department of Chemistry, University of California Davis, One Shields Avenue, Davis, CA 95616.

Three recombinant microbial CMP-sialic acid synthetases [EC 2.7.7.43] were cloned from *Neisseria meningitidis* group B, *Streptococcus agalactiae* serotype V, and *Escherichia coli* K1, respectively, and expressed as C terminal His_6-tagged proteins in *E. coli*. Their biochemical properties were compared. Combined with a recombinant sialic acid aldolase [EC 4.1.3.3] cloned from *E. coli* K12, these CMP-sialic acid synthetases were evaluated on their abilities in the synthesis of CMP-sialic acid analogs in a one-pot two-enzyme system. The *N. meningitidis* synthetase was shown to have the highest expression level, the most flexible substrate specificity, and the highest catalytic efficiency among three synthetases. CMP-sialic acid analogs were synthesized in preparative scales from their 5- or 6-carbon sugar precursors using the *N. meningitidis* synthetase and the aldolase. The *N. meningitidis* synthetase was also used in a one-pot preparation of sialates with the aldolase and a sialyltransferase cloned from *Pasteurella multocida*.

(114) **In vitro Glycoprotein Remodeling of Recombinant Soluble Human Complement Receptor Type 1, sCR1.**

Lawrence J. Thomas[1], Krishnasamy Panneerselvam[2], David T. Beattie[1], Michele D. Picard[1], Bi Xu[1], Charles W. Rittershaus[1], Henry C. Marsh[1], Russell A. Hammond[1], Jun Qian[2], Tom Stevenson[2], David Zopf[2] and Robert J. Bayer[2]
[1] AVANT Imunotherapeutics, Inc. Needham MA, [2] Neose Technologies, Inc. Horsham, PA and San Diego, CA.

Soluble complement receptor (sCR1) is a glycoprotein intended for use as a drug to treat ischemia-reperfusion injury and other complement-mediated diseases and injuries. We have introduced sialyl Lewis x moieties in vitro onto the N-glycan chains of sCR1 overexpressed in CHO using recombinant α 2-3 sialyltransferase ST3Gal-III and the α 1-3 fucosyltransferase FT-VI at 10 gram scale. The product has approximately 14 N-glycan chains per sCR1 molecule, nearly all of whose antennae terminate with sialyl Lewis x moieties. The remodeled sCR1 exhibited a two-fold increase in area under the intravenous clearance curve in a rat pharmacokinetic model. Compared to a similar molecule produced in the LEC11 cell line which expresses an active endogenous fucosyltransferase, it contained twice the number of sialyl Lewis x moieties per mole of glycoprotein, and exhibited a 10-fold increase in affinity for E-selectin in an in vitro binding assay.

(115) **Synthesis of Sialoside Inhibitors of Myelin-Associated Glycoprotein (Siglec-4)**

Tasneem Islam, Ola Blixt, Hiroaki Tateno and James C. Paulson
The Scripps Research Institute, Departments of Molecular Biology and Molecular and Experimental Medicine, La Jolla, California 92037.

Following nerve injury, axons in the central nervous system (CNS) lose their ability to regenerate. Evidence suggests that at least three components of the injured CNS inhibit neurite (axon) outgrowth: myelin-associated glycoprotein (MAG), Nogo (a myelin protein) and chondroitin sulfate proteoglycan (CSPG). MAG, also called Siglec 4, is a member of the sialic acid-dependent Ig-family member lectins (Siglecs). This quantitatively minor component of the proteins in central and peripheral nervous system myelin is involved in stabilization of myelin-axon interactions, control of axonal cyto-architecture and inhibition of nerve regeneration. MAG binds preferentially to the two gangliosides, GD1a and GT1b, bearing the NeuAcα2-3Galβ1-3GalNAc at the non-reducing terminus. Support for the role of these gangliosides as physiological MAG ligands is derived from nerve regeneration studies in vitro. Neonatal rat cerebellar granule cells showed an extensive meshwork of axons on control surface, but decreased growth on surfaces with MAG. This MAG-mediated neurite outgrowth inhibition is reversed by blocking glycosphingolipid biosynthesis and by antibodies to GD1a and GT1b.[1]
Studies on the carbohydrate binding specificity of MAG for gangliosides revealed that glycolipids containing the disialyl tetrasaccharide sequence NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc were more potent MAG binding ligands than GD1a or GT1b.[2] Recently, it was shown that a synthetic threonine glycoside of the same sequence (NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAcαThr(OCH3)) was a highly potent inhibitor of MAG in a multivalent binding assay.[3] Based on this study, we have embarked on a systematic evaluation of analogs of this inhibitor by modifying the C-9 and C-5 positions of one or both NeuAc residues to further increase affinity for MAG. We report here our exploration of these monovalent MAG-binding glycans. Such compounds may be useful tools to investigate the role of MAG as an inhibitor of nerve regeneration.
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(116) **Synthesis of Major Glycolipid Antigens of *Borrelia burgdorferi* for use in Conjugate Vaccines Against Lyme Disease**

Vince Pozsgay, Joanna Kubler-Kielb, Bruce Coxon, Gil Ben-Menachem and Rachel Schneerson

National Institute of Child Health and Human Development, National Institutes of Health, 31 Center Dr. MSC 2423 Bethesda, MD, 20892-2423.

Lyme disease is a multisystem infection that is spreading in the Northeastern United States. The causative agent of this disease is the spirochete *Borrelia burgdorferi* that is transmitted to mammals through the tick vector. Despite growing concerns caused by this bacterium, no vaccine is available for the prevention of Lyme disease in humans in the US. We showed (Ben-Menachem et al., *PNAS*, 100, 7913, 2003) that *B. burgdorferi* synthesizes cholesteryl 6-O-palmitoyl and 6-O-oleoyl-β-D-galactopyranoside as its major glycolipids in approximately equal portions. It was also suggested that these glycolipids may assume the function of the bacterial lipopolysaccharides. To provide these antigens in quantities suitable for studying their antigenicities and immunogenicities, we have developed synthetic routes for their production. Here, we wish to report on the chemical synthesis of these and related glycolipids, and outline our approach to their protein conjugates for use as experimental vaccines. For the synthesis of the target glycolipids, we employed a pivaloylated galactosyl donor, that was stereoselectively coupled with cholesterol. Next, the acyl protecting groups were removed from the galactose residue. In the subsequent stages, the galactose moiety was regioselectively protected through several steps to provide a 6-O unprotected intermediate which was acylated with either palmitic acid or oleic acid. Subsequently, the protecting groups were selectively removed from the O-2,3, and 4 positions of the galactose unit to provide the targeted glycolipids. The synthesis of related glycolipids will also be described.

(117) **An Active Site Mutant of the Retaining Glycosyltransferase LgtC from *Neisseria meningitidis* Contains a Covalently Bound Galactosyl-enzyme Intermediate and Reveals an Alternative Candidate Catalytic Nucleophile**

Luke L. Lairson[1], Cecilia P.C. Chiu[2], Hoa D. Ly[1], Shouming He[1], Warren W. Wakarchuk[3], Natalie C.J. Strynadka[2] and Stephen G. Withers[1,2]

[1] Department of Chemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z1, [2] Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3 , [3] Institute of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6, .

While the mechanistic strategies used by glycosidases to catalyse glycosidic bond hydrolysis are fairly well understood on both a structural and chemical level, the characterization and mechanistic understanding of the glycosyltransferases responsible for glycoside bond formation has lagged far behind. This lack of insight is particularly apparent amongst the retaining glycosyltransferases. By direct comparison to retaining glycosidases, the mechanism of retaining glycosyltransferases has been proposed to be that of a double displacement mechanism involving a covalently bound glycosyl-enzyme intermediate, demanding the existence of an appropriately positioned nucleophile within the active site. However, the conclusive identification of a catalytic nucleophile and observation of a covalent intermediate has yet to be reported for any retaining transferase despite exhaustive studies using techniques that have been successfully applied to the characterization of retaining glycosidases. LgtC, a GT family 8 α-1,4-galactosyltransferase from *Neisseria meningitidis*, catalyzes the transfer of galactose from UDP galactose to terminal lactose-containing acceptor sugars with net retention of anomeric configuration and is therefore a retaining glycosyltransferase. To investigate the potential role of discrete nucleophilic catalysis suggested by the double displacement mechanism generally proposed for retaining glycosyltransferases, the side chain amide of Gln189, which is suitably positioned to act as the catalytic nucleophile of LgtC, was substituted with the more nucleophilic carboxylate-containing side chain of glutamate in the hope of accumulating a glycosyl-enzyme intermediate. The resulting mutant was subjected to kinetic, mass spectrometric and X-ray crystallographic analysis. While the KM for UDP Gal is not significantly altered, the kcat was reduced to 3% of that of the wild type enzyme. ESMS analysis revealed that a steady state population of the Q189E variant contains a covalently bound galactosyl moiety. LC/MS analysis of fragmented proteolytic digests identified the site of labeling not as Glu189, but surprisingly as the sequentially adjacent, carboxylate-containing residue within the active site, Asp190. However, the side chain carboxylate of Asp190 is located 8.9 ≈ away from the donor substrate in the available ground state crystal structure. Kinetic analysis of an Asp190Asn mutant at this position revealed a kcat value 3000 fold lower than that of the wild type enzyme. A 2.6 ≈ crystal structure of the Gln189Glu mutant with UDP 2FGal bound in the active site revealed no significant perturbation of the mode of donor sugar binding nor of active site configuration compared to that of the wild type enzyme. This suggests that the site of labeling is not simply the result of a structural perturbation of the active site resulting from the introduced mutation. It also implies that if Asp190 does in fact play the role of catalytic nucleophile, a significant conformational change must occur during catalysis in order for this residue to become appropriately positioned within the active site. This is the first trapping of an intermediate in the active site of a retaining glycosyltransferase and, although not conclusive, implicates Asp190 as an alternative candidate catalytic nucleophile.

(118) **Kinetic Studies of Endo-β-galactosidase by a Novel Colorimetric Assay and Synthesis of Poly-*N*-acetyllactosamines Using its Transglycosylation Activity**

Takeomi Murata, Takeshi Hattori, HIroki Honda, Satoshi Amarume and Taichi Usui

Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan.

Novel chromogenic substrates for endo-β-galactosidase were designed on the basis of the structural features of keratan sulfate. Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ-*p*NP (**2**), which consists of two repeating units of *N*-acetyllactosamine, was enzymatically synthesized by consecutive additions of GlcNAc and Gal residues to *p*-nitrophenyl β-*N*-acetyllactosaminide. In a similar manner, GlcNAcβ1,3Galβ1,4GlcNAcβ-*p*NP (**1**), GlcNAcβ1,3Galβ1,4Glcβ-*p*NP (**3**), Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ-*p*NP (**4**), Galβ1,3GlcNAcβ1,3Galβ1,4Glcβ-*p*NP (**5**), and Galβ1,6GlcNAcβ1,3Galβ1,4Glcβ-*p*NP (**6**) were synthesized as analogs of **2**. Endo-β-galactosidases released GlcNAcβ-*p*NP or Glcβ-*p*NP in an endo-manner from each substrate. A colorimetric assay for endo-β-galactosidase was developed using the synthetic substrates on the basis of the determination of *p*-nitrophenol liberated from GlcNAcβ-*p*NP or Glcβ-*p*NP formed by the enzyme through a coupled reaction involving β-NAHase or β-D-glucosidase. Kinetic analysis by this method showed that the value of V_{max} /K_m of **2** for *Escherichia freundii* endo-β-galactosidase was almost equal to that for keratan sulfate, indicating that **2** is very suitable as a sensitive substrate for analytical use in an endo-β-galactosidase assay. In addition, the hydrolytic action of the enzyme toward **2** has shown to be remarkably promoted by the presence of 2-acetamide group adjacent to *p*-nitrophenyl group in comparison with **4**. In addition, enzymatic synthesis of GlcNAc-terminated poly-*N*-acetyllactosamine β-glycosides GlcNAcβ1,3(Galβ1,4GlcNAcβ1,3)$_n$Galβ1,3GlcNAcβ-*p*NP (n= 1-4) has been demonstrated using a transglycosylation reaction of *E. freundii* endo-β-galactosidase. The enzyme catalyzed a transglycosylation reaction on **1**, which served both as a donor and an acceptor, and converted **1** into *p*-nitrophenyl β-glycosides GlcNAcβ1,3(Galβ1,4GlcNAcβ1,3)$_1$Galβ1,4GlcNAcβ-*p*NP (**7**), GlcNAcβ1,3(Galβ1,4GlcNAcβ1,3)$_2$Galβ1,4GlcNAcβ-*p*NP (**8**), GlcNAcβ1,3(Galβ1,4GlcNAcβ1,3)$_3$Galβ1,3GlcNAcβ-*p*NP (**9**) and GlcNAcβ1,3(Galβ1,4GlcNAcβ1,3)$_4$Galβ1,4GlcNAcβ-*p*NP (**10**). When **7** was used as an initial substrate, it led to the preferential synthesis of nonasaccharide β-glycoside **9** to heptasaccharide β-glycoside **8**. This suggests that **9** is directly synthesized by transferring the tetrasaccharide unit GlcNAcβ1,3Galβ1,4GlcNAcβ1,3Gal to non-reducing end GlcNAc residue of **7** itself. The efficiency of production of poly-*N*-acetyllactosamines by *E. freundii* endo-β-galactosidase was significantly enhanced by the addition of BSA and by a low temperature condition. Resulting **7** and **8** were shown to be useful for studying endo-β-galactosidase-catalyzed hydrolytic and transglycosylation reactions.

(119) **A Study of Fucoidan Oligosaccharides: Synthesis and Characteristics of Sulfated Fucotriosides**

Tetsuo Oh-ishi[1], Kenzo Ohtsuki[1] and Naohiko Morishima[2]

[1] Laboratory of Biochemistry and Signal Biology, Graduate School of Medical Sciences, Kitasato University, Sagamihara 228-8555, Japan, [2]

Laboratory of Biochemistry, School of Nursing, Kitasato University, Sagamihara 228-0829, Japan.

Sulfated fucans constitute a class of polysaccharides mainly consisting of sulfated L-fucopyranose, and occur in brown algae and marine invertebrates. The sulfated fucans extracted from brown algae are often called fucoidans, which are usually more complex in structure than those from marine invertebrates. Fucoidans are known to have many biological activities, such as anticoagulant, antiproliferative, anti-inflammatory, and antiviral effects, and inhibitory effect on enzyme activities of some phospholipases. However, as most biological properties have been examined with crude fucoidans whose structures were not defined, the relationships between biological activity and molecular structure have not well interpreted so far. Therefore, many of recent studies on fucoidans have focused upon the repeating unit structure, or upon the dependence of activities on the degree of sulfation. Thus, we have considered that these biological activities may not only be a function of the degree of sulfation but that of the structural features such as sulfated positions and/or sequence of fucose chains. Under the hypothesis, we have synthesized various sulfated fucotriosides, which are subject to assay of certain biological activities. Synthesis of the sulfated trisaccharides were performed via stepwise glycosylation, followed by sulfation. As the glycosyl acceptors, to be residues at each reducing end of the trisaccharides, 2-methoxyethyl 2,4-, and 2,3-di-O-benzyl-beta-L-fucopyranosides were prepared from L-fucose through seven and eight steps, respectively. After acetylation of the hydroxyl group of each di-O-benzyl derivative, the 2-methoxyethyl group was removed with the aid of titanium(IV) chloride, and subsequent fluorination with DAST gave 3-O-acetyl-2,4-di-O-benzyl-, or 4-O-acetyl-2,3-di-O-benzyl-L-fucopyranosyl fluoride. Glycosylation of the above glycosyl acceptors with the fucosyl fluorides using tin(II) chloride, silver perchlorate, and MS4A in dichloromethane almost exclusively afforded the alpha-linked disaccharides in yields of 60-90%. Subsequently, the deacetylated fucobiosides were stereoselectively glycosylated in the same manner to give the trisaccharide derivatives in yields of 55-88%. Removal of the protective groups furnished 2-methoxyethyl glycosides of the following alpha-linked fucotrioses; Fuc(1-3)Fuc(1-3)Fuc, Fuc(1-4)Fuc(1-3)Fuc, and Fuc(1-3)Fuc(1-4)Fuc. Another alpha-linked trisaccharide, Fuc(1-3)[Fuc(1-2)]Fuc, being a constituent at the branching point of fucoidans, was also synthesized with a simultaneous glycosylation at 2- and 3-positions of 2-methoxyethyl 4-O-benzyl-beta-L-fucopyranoside. Sulfation with sulfur trioxide-trimethylamine complex in N,N-dimethylformamide gave the sulfated fucotriosides. Inhibitory effect of these compounds on enzyme activities is discussed on the basis of structural specificities.

(120) **A Large Family of β-1,3-glycosyltransferases: Cloning a β-1,3-*N*-acetylglucosaminyltransferase Synthesizing Polylacnac.**

Hiroyasu Ishida[1,2], Akira Togayachi[1], Tokiko Sakai[1,3], Toshie Iwai[1], Takashi Sato[1], Toru Hiruma[1,4], Kumi Ishikawa[1], Nobuo Nakanishi[1], Niro Inaba[1,5], Takashi Kudo[1,6], Masanori Gotoh[1], Hiroko Iwasaki[1] and Hisashi Narimatsu[1]

[1] Glycogene Function Team, Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), [2] Medical Sciences for Control of Pathological Processes, Graduate School of Comprehensive Human Sciences, Tukuba University, [3] Central Research Laboratories, Seikagaku Corporation, [4] Fujirebio, Inc., [5] JGS Japan Genome Solutions, Inc., [6] Institute of Basic Medical Sciences, Department of Anatomy and Embryology, University of Tsukuba.

The glycosyltransferases can be grouped into functional families based on their sequence similarities, which reflect their enzymatic characters; donor specificity, acceptor specificity, and specific linkage between donor and acceptor. For these years, our group has been pursuing the cloning of a set of human genes encoding glycosyltransferases transferring sugars via a β-1,3-linkage. They are divided into β3Gal-T, β3GalNAc-T, and β3Gn-T groups. The members form a large gene family, and each member shares a β3-GT motif. In this study, we identified two novel members of the β3Gn-T family having the motifs, by in silico methods. Two novel β3Gn-Ts were cloned from human cell lines and named β3Gn-T7 & -T8 based on their positions in a phylogenetic tree and their enzymatic activity. The β3Gn-T7 transcripts were considerably high in lung, stomach, colon, pancreas, spleen, and placenta. The β3Gn-T8 transcripts were considerably high in small intestine, pancreas, spleen, bone marrow, and skeletal muscle. The expression levels of β3Gn-T7 in cancer tissue were down-regulated compared to those in the normal tissues. By transfection experiments and in vitro enzymatic analysis, β3Gn-T8 was found to catalyze the synthesis of polylactosamine chains. Its specificity for acceptor substrates is different from the other members which also synthesize polylactosamine chains. Fine specificities of each enzyme will be discussed.

(121) **Efficient Stereoselective Synthesis of *N*-Linked Glycopeptides by N-Glycosylation of Primary Amide Groups.**

Hiroshi Tanaka, Yuki Iwata, Daisuke Takahashi, Masaatsu Adachi and Takashi Takahashi

Department of Applied Chemistry, Graduate School of Science and Engineering Tokyo Institute of Technology, 2-12-1 Ookayama, Meguro, Tokyo 152-8552, Japan.

Glycoproteins play an important role in essential biological processes such as cell recognition, cell aadhesion, immunogenic recognition, and so on and contain *O*- or *N*-linked glycosides. In order to study these mechanisms, synthetic glycopeptides are needed as chemical probes. Therefore, an effective methodology for the synthesis of the various glycosyl peptides is required.

The N-glycosides are attached to asparagines at the γ position through β-glycosidic bond. Commonly, synthesis of linking asparagine and oligosaccharide has been achieved by amidation of asparaginic acid with the glycosyl amine or its equivalent. However, the β-glycosyl amine is sufficiently unstable not only to epimerize at the anomeric position, but also to hydrolyze to the lactol during the reaction. Furthermore, cyclization could occur to provide aspartimide during the amidation. In order to avoid these problems, direct N-glycosylation of the primary amides with glycosyl donors would be an alternative and effective way. However, it is worth nothing that the nitrogen of the amide groups showed very poor nucleophilicity towards glycosylation. In fact, there is an only report for the chemical synthesis of N-glycoside by N-glycosylation till today. In this report, we describe the simple and efficient method for preparation of glycosyl amino acids and peptides by N-glycosylation of primary amides.

We first examined glycosylation of *N*-silyl acetamide with galactosyl fluoride and imidate, and thiogalactoside. Glycosylation of 3 equivalents *N*-silyl acetamide with the donors in the presence of appropriate activators was achieved. The only galactosyl imidate was converted to the desired *N*-glycosyl acetamide in moderate yield. Further optimization of the reaction conditions utilizing three activators (TMSOTf, TESOTf, TBSOTf), five solvents (CH_2Cl_2, diethylether, acetnitrile, THF, toluene) and two acceptors (*N*-silyl acetamide and acetamide) were examined. The N-glycosylation under 30 different conditions was achieved in parallel fashion utilizing an automated parallel synthesizer and automated column chromatography. We found that the N-glycosylation of *N*-silyl acetamide and acetamide in the presence of TMSOTf in acetnitrile proceeded to give the desired *N*-glycosyl acetamide in 86% and 74% yields, respectively. To improve the reaction conversion, we treated acetamide with an excess of galactosyl imidate. However, the yield was reduced to be 42% because N-glycosylation of the released trichloroacetamide underwent N-glycosylation to provide *N*-glycosyltrichloroacetamide.

To overcome the problem, we next investigated glycosylation of acetamaide with *N*-aryl trifluoroimidates. Steric hindrance of the *N*-substitution could prevent the formation of *N*-glycosidic bond with the release *N*-ary acetamide. Glycosylation of acetamide with 1.5 equivalents three galactosyl imidates (*N*-phenyl, methoxyphenyl, fluorophenyl), in the presence of three activators (TMSOTf, TESOTf, TBSOTf), and in three solvents (acetnitrile, propionitrile, nitromethane) were examined. The glycosylation under 27 different reaction conditions was accomplished utilizing the automated synthesizer. We found that glycosylation of acetamide with *N*-phenyltrifluoroimidate in the presence of a catalytic amount of TMSOTf at room temperature in nitromethane provided the *N*-glycosyl acetamide in 98% yield.

In this poster session, we will also present the synthesis of *N*-glycosyl asparagines and peptides by the N-glycosylation.

(122) **Synthesis of Actinoflavoside; Glycosyldation of a New Flavanone with D-Ristosamine**

Masanori Yamaura, Katsuhiko Suzuki, Takeshi Tsuruga, Kyouko Hiranuma and Yuusuke Watanabe

Department of Enviromental Science, Faculty of Science and Engineering, Iwaki Meisei University 5-5-1, Iino, Chuohdai, Iwaki-shi, 970-8551, Fukushima, Japan.

Actinoflavoside, 7-{2, 3, 6-trideoxy-3-[3-(*R*)-hydroxy-2-(*R*)-methyl-butanoic acid]amino-α-D-*ribo*-hexopyranosyl}-8 -hydroxy-5-hydroxymethyl-2-phenyl-chroman-4-one, which was isolated from the culture fluid of ocean bacillus CNB-689 of the *Streptomyces* genus, was the rare example as flavonoid glycosides[1]. We interested in this compound

(isolated from the ocean bacillus), and started the total synthesis for examination of its biological activity and absolute configuration in detail. Recently, we accomplished the convenient synthesis of 7, 8, -dihydroxy-5-hydroxymethyl-2 -phenyl-chroman-4-one, the aglycon part of Actioflavoside[2]. In this session, we would like to discuss the stage toward the synthesis of Actioflavoside. As a glycosyl acceptor,Å@7-hydroxy-8-acetoxy-5-(*tert*-butyl-dimethyl-silanyloxymethyl) -2-phenyl-chroman-4-one was synthesized. This aglycon will be hopeful of the regoselectivity and solubillty on the glycosyldation. As a glycosyl donor, two D-ristosamine derivatives were prepared. However, glycosyldation using 3-acetamido-2, 3, 6-trideoxy-4-*O*-benzoyl-α-D-*ribo*-hexopyranosyl trichloroacetimidate was unsuccessful. In this case, the donor was transformed into the corresponding glucal via oxazoline. It seems that the nucleophilicity of the carbonyl oxygen at the acetamido group is stronger than that of in the aglycon.Å@On the other hand, glycosidation using *O*-3 -benzyloxycarbonyamino-2, 3, 6-trideoxy-4-*O*-benzoyl- α-D-*ribo*-hexopyranosyl trichloroacetimidate as a donor smoothly proceeded to give the corresponding flavonoid glycoside . We will discuss detail of this reaction, and in addition, it is also mentioned on the coupling with an optical active side chain. [1] Zhi-Dong Jiang, Paul R. Jensen, William Fenical, *Tetrahedron Lettera*, 29, 5065-5068 (1997). [2] Katsuhiko Suzuki, Takeshi Tsuruga, Kyoko Hiranuma, Masanori Yamaura, *Synlett*, 1, 116-118(2004).

(123) **Combinatorial Synthesis of Natural Products: Automated Parallel Synthesis of Dimeric Lewis X Library by One-Pot Sequential Glycosylation**

Takashi Takahashi, Nobuatsu Matoba, Hirokazu Tsukamoto, Hisami Takimoto and Hiroshi Tanaka

Department of Applied Chemistry, Graduate School of Science and Engineering, Tokyo Institute of Technology, 2-12-1 Ookayama, Meguro, Tokyo 152-8552, Japan..

Oligosaccharides on cell surface play important roles in many biological processes. Most biologically active oligosaccharides are rare, and are difficult to purify. Therefore, the chemical synthesis of such oligosaccharides would strongly assist in the elucidation of their structure-activity relationships. However, the synthesis of oligosaccharides still requires experts in organic synthesis in comparison with the synthesis of peptides and oligonucleotides. Herein we report an automated parallel solution-phase synthesis of a protected dimeric Lewis X library by one-pot glycosylation.

Dimeric Lewis X is an important tumor-associated antigen and is composed of β(1,3) and β(1,4) linked hexasaccharide backbone attached via two α(1,3) linked branching saccharides. Our strategy for the synthesis of the dimeric Lewis X epitope involves one-pot glycosylation using four building blocks. The one-pot glycosylation was initiated by chemo- and regio-selective glycosylation of 2-*N*-phthaloyl thioglucosamine at the C4 position with galactosyl fluoride attached with a branched trisaccharide at the C3 position. Activation of the resulting thioglycoside and coupling with the glycosyl acceptor, followed by α-selective glycosylation of the remaining C3 hydroxyl group of the glucosamine unit with thiofucoside provides the protected octasaccharide in one-pot. The *N*-phthaloyl group reduces the reactivity of the C3 hydroxyl group of the glucosamine to promote regio-selectively glycosylation at the C4 hydroxyl group due to steric hindrance, and generates the 1,2 trans glycosidic bond in the second glycosylation. The branched tetrasaccharide can be prepared by one-pot three-step glycosylation in the same manner using the glycosyl bromide, the *N*-phthaloyl thioglucosamine, the galactosyl fluoride, and thiofucoside. Various glycosyl halides, glycosyl acceptors and thioglycosides would be adaptable to the one-pot glycosylation instead of building blocks to provide combinatorial oligosaccharide libraries based on the structure of dimeric Lewis X.

The one-pot glycosylation for the synthesis of the protected octasaccharide was examined. Sequential coupling reaction of the four building blocks with the appropriate activators such as AgOTf and DMTST provided the glycosyl fluoride in 56% overall yield based on the *N*-phthaloyl thioglucosamine. The following one-pot glycosylation using the four building blocks with the appropriate activators such as $HfCp_2Cl_2$/AgOTf and NIS/TfOH afforded the protected octasaccharide in 75% yield based on the lactose moiety.

Next, we conducted an automated parallel synthesis of a small combinatorial library containing the protected azido-bearing di- to hepta-saccharides varying in the number of the branched fucoses and the length of the backbone. Linear type one-pot glycosylation using the three glycosyl donors, glucosamine and two acceptors provides 6 oligosaccharides. Further incorporation of fucoside to oligosaccharides at the remaining hydroxyl group affords 6 branched oligosaccharides. The library synthesis was achieved utilizing an automated synthesizer (L-COS^{TM}) in which controlled stirring, reaction temperature, and rate of addition of reagents can be programmed. It took eight hours to finish the program after set-up of all reagents. Purification of the twelve crude compounds using silica gel chromatography and a gel permeability chromatography provided twelve protected oligosaccharides in 22-46% overall yields based on azido alcohols

(124) **Synthesis of 1-*S*-2-*O*-Benzylidene Sugar and Its Application to Stereoselective Glycosylation Reaction**

Hisato Nonaka, Katsuhiko Suzuki And Masanori Yamaura

Department of Environmental Science, Faculty of Science and Engineering, Iwaki Meisei University, 5-5-1 Iino, Chuohdai, Iwaki-shi, Fukishima 970-8551, Japan.

Benzylidene type acetal is useful for the protection of adjoining hydroxyl groups, and the reactions including the ring-opening is used frequently. In our recent research, a practical synthesis of the 1,2-*O*-benzylidene sugars and its the regioselective ring-opening was accomplished. Thus, the 1,2-*O*-benzylidene sugars were added to the useful synthone in the carbohydrate chemistry [1]. On the other hand, 1,2-*O*-benzylidene group linking an anomeric position may be candidate for the glycosyl donor as like 1,2-*O*-orthoester. However, the utilization of the anomeric benzylidene group for the glycosylation has not been investigated. We thought that the 1,2-benzylidene donor having bicyclic 1,2-*cis*-linkage suit for the formation of 1,2-*trans*-glycoside, because of the rigid conformation. Then, we started synthesis of the 1,2-benzylidene sugar as a new glycosyl donor. In this session, we would like to report the synthesis of 1-*S*-2-*O*-benzylidene sugar and its application to stereoselective glycosylation. First, 3,4,6-Tri-*O*-benzoyl-1,2-di-*O*-*p*-methoxythiobenzoyl-D-mannopyranose was derived from 3,4,6-Tri-*O*-benzoyl-D-mannopyranose by the thioesterification with *p*-methoxydithiobenzoic acid using DCC or EDC. 1-*S*-2-*O*-benzylidene function was constructed by the reductive cyclization method. The 1,2-thiobenzoate derivatives were treated with HBr/AcOH and the product was subsequently reduced by the addition of $NaBH_3CN$ to give 3,4,6-Tri-*O*-benzoyl-1-*S*-2-*O*-benzylidene-D-mannopyranose in good yield. The 1-*S* position of 1-*S*-2-*O*-benzylidene was effectively activated by Phenylselenenyl triflate (PhSeOTf), and the α-selective glycosylation with various primary and secondary sugar alcohols was achieved. We will report the stereoselectivity and detail of this reaction. [1] Suzuki, K., Mizuta, T., Yamaura, M., *J. Carbohydr. Chem.*, *22*, 143-147 (2003). Suzuki, K., Nonaka, H., Yamaura, M., *Tetrahedron Lett.*, *44*, 1975-1977 (2003). Suzuki, K., Nonaka, H., Yamaura, M., *J. Carbohydr. Chem.*, in press.

(125) **Discovery of Potent and Selective alpha-Fucosidase Inhibitors**

Chun-Hung Lin, Chuan-Fa Chang, Chung-Yi Wu and Chin-Wen Ho

Institute of Biological Chemistry and Genomic Research Center, Academia Sinica,

No.128 Academia Road Section 2, Nan-Kang, Taipei, 11529, Taiwan.

Glycosidase inhibitors have shown great medicinal and pharmaceutical values as best exemplified by the therapeutic treatment of influenza virus and non-insulin-dependent diabetes. We have recently developed a diversity-oriented synthesis in microplates for high throughput screening in situ without protecting group manipulation and product isolation, and have identified the most potent and selective inhibitors against the alpha-fucosidase from bovine kidney as well as Corynebacterium sp. This strategy is based on a rapid screening for an optimal aglycon attached to the fuconojirimycin (FNJ)-based structure that mimics the transition state of enzymatic glycoside cleavage. The library screened for two alpha-fucosidases generated different groups of structures, indicating that our approach is effective and simple for identification of potent inhibitors that are enzyme-specific, and have no or little inhibition activity against other glycosidase members (e.g. glucosidase, galactosidase and mannosidase). Furthermore, the same method also led to the discovery of slow and tight binding inhibitors of alpha-fucosidase. The introduction of a hydrophobic moiety to the iminocyclitol core not only resulted in the time-dependent inhibition, but also greatly enhanced the inhibitory potency to give the Ki* value in the pM range. The time-dependent inhibition displays the progressive tightening of enzyme-inhibitor complex from a low nanomolar Ki to picomolar Ki* value. Particularly the best one with a Ki* of 0.46 pM represents the most potent glycosidase inhibitor to date. The intrinsic fluorescence of alpha-fucosidase is enhanced as a function of inhibitor

concentration indicating a subtle conformational change in the enzyme, which is correlated with the isomerization of Ki to Ki*. This work clearly demonstrates the effectiveness of our combinatorial approach to the rapid discovery of potent inhibitors. References (1) Wu, C.-Y., Chang, C.-F., Chen, J. S.-Y., Wong, C.-H., Lin, C.-H. (2003). Angew. Chem. Int. Ed. 42, 4661-4664. (2) Chang, C.-F., Ho, C.-W., Wu, C.-Y., Chou, T.-A., Wong, C.-H., Lin, C.-H. (2004) Chem. Biol. in press.

(126) **Substrate Based Metabolic Engineering of Cell Surface Sialic Acids: Synthesis and Applications of Novel *N*-acylmannosamine Analogs Possessing Pendant Thiol Groups as Metabolic Precursors**

S-Gopalan Sampathkumar, Mark B Jones and Kevin J Yarema
Dept of Biomedical Engineering, Johns Hopkins University, 3400 N Charles St, Clark Hall Rm 107, Baltimore MD 21218.

The plasma membrane of a eukaryotic cell is decorated with various glycoconjugates, including N- and O- linked glycoproteins, glycolipids, glycosylphosphatidylinositol (GPI) anchors and proteoglycans, termed collectively as 'glycocalyx'. In mammalian cells sialic acids, a family of nine carbon sugars, are found on the non-reducing termini of these glycans. Surface sialoglycoconjugates are critical for various biological events such as signal transduction, leukocyte recruitment, fetal development, host-pathogen interaction and cancer metastasis. Endogenous sialic acid (Neu5Ac) is produced from N-acetylmannosamine (ManNAc) and the biosynthetic pathway has a remarkable ability to accept non-natural, externally supplied ManNAc analogs as metabolic substrates. The broad substrate specificities of the pathway enzymes allow the structure of the surface sialic acids to be metabolically engineered starting from simple non-natural precursors of ManNAc. Up to now, this 'sialic acid engineering' methodology has been used to alter the expression of natural sialic acids as well as install non-natural forms of this sugar bearing extended alkyl N-acyl groups as well as N-acyl chains bearing hydroxyl, ketone, and azide functionalities.

The present study reports our efforts towards introducing additional functionalities and expanding the scope of the cell surface engineering methods. Accordingly, we have synthesized several novel peracetyl-N-acylmannosamine analogs possessing (a) pendant thiol groups on the N-acyl moiety (acetylthioalkyl-, methylthioalkyl- and methylsulfinylthio-) and (b) the 6-acetylthioManNAc. The results of the studies on their cellular uptake, toxicity, efficiency of conversion to modified sialic acids and cell surface expression will be presented. One of the analogs, Ac_5ManNTGc, a sulfur substituted analog of the natural N-glycolyl-mannosamine (ManNGc), resulted in efficient cell surface expression of Neu5TGc, a novel thiol modification of sialic acid. The cell surface expression of the SiaSH was confirmed by selective labeling of the thiol groups followed by flow cytometry. Practical uses for thiol-modified sialic acids arise from the growing realization that cysteine residues of cell surface proteins form permanent or transient disulfide bonds with proteoglycans of the ECM and play a major role in tissue formation and maintenance. Consequently, the ability to modulate these interactions hold value in diverse applications ranging from altering the metastatic potential of cancer cells to providing new forms of cell adhesion of value to tissue engineering efforts.

(127) **In vivo Preparation of Homogeneous Glycoforms in *Escherichia Coli***

Sarah R. Hanson, Ran Xu, Der-Ren Hwang, Zhiwen Zhang, Yu-Ying Yang, Carmen Galan, Peter G. Schultz and Chi-Huey Wong
The Scripps Research Institute; Department of Chemistry; 10550 North Torrey Pines Road; La Jolla, CA 92037.

Glycosylation is a prevalent and vast posttranslational process capable of augmenting and modulating protein functions, such as folding, stability, localization, intrinsic activity, and bimolecular interaction and recognition events. Efficient synthesis of high-purity, homogenous glycoproteins is essential for both the study and understanding of unique protein glycoforms and for the manufacture of therapeutically relevant forms. Herein, suppressor tRNA technology was examined as a means to incorporate the major mucin glycoprotein core, Galactosamine-α-O-L-theronine (GalNAc-α-Thr), and related unnatural amino acid analogues into proteins of interest. In vivo suppressor tRNA technology relies on the evolution of a suppressing tRNA aminoacyl synthetase-tRNA pair that selectively charges an unnatural amino acid, without any cross reactivity with endogenous pairs. Then, during protein biosynthesis, the evolved pair can site-specifically insert an unnatural amino acid in response to nonsense or four-base codons that have been engineered at permissive positions in proteins of interest. In the case of GalNAc-α-Thr, an orthogonal pair was derived from the *Methanococcus jannaschii* tyrosinyl-tRNA aminoacyl synthetase (MjTyrRS) and Tyr-tRNA to suppress the amber codon, TAG, thus allowing for the homogneous expression of glycoproteins in *E. coli*. Incorporation of GalNAc-α-Thr into therapeutic targets of interest, including interleukin and Ribonuclease A, will allow for the study of the specific role that glycosylation plays in these proteins.

(128) **Structural Determinants of the Glycosylation of Thrombospondin Type 1 Repeats**

Boris Macek[1], Dominique Klein[2], Daniel Hess[2] and Jan Hofsteenge[2]
[1] Institute for Medical Physics and Biophysics, University of Muenster, Germany, [2] Friedrich Miescher Institute, Basel, Switzerland.

Thrombospondin type 1 repeats (TSRs) are modules of approximately 60 amino acid length that are important for the biological activity of a variety of proteins (e.g. extracellular matrix components, proteases, complement factors and axonal guidance molecules). They consist of three antiparallel strands, and contain a core formed by alternating side chains from Trp- and basic residues (mainly Arg). The core is capped at both ends by conserved disulfide bridges (CWR stack). TSRs contain two kinds of glycans: C-mannosyl moieties attached to the Trp residues in the CWR stack, and the disaccharide Glc-Fuc- on a Thr or Ser residue in the loop connecting the first two strands. C-mannosylation is carried out by a protein C-mannosyltransferase activity (PCMT) in the lumen of the rough endoplasmic reticulum. The enzyme uses Man-P-Dol as the sugar donor, and modifies most efficiently Trp residues in the motif W-x-x-W. In certain proteins, exemplified by RNase 2, C-mannosylation occurs exclusively on the first Trp, and the presence of the second Trp (or with lower efficiency a Phe residue) is required for this. TSRs often contain repeated C-mannosylation motifs, i.e. W-x-x-W-x-x-W. Surprisingly, in TSRs all three Trps can be C-mannosylated, implying that in these modules a Trp at the +3 position is not required. O-fucosylation in TSRs differs from that found in EGF repeats, with respect to the glycan (Glc-Fuc-), the consensus sequence around the site of attachment (W-X2-W-X2-C-X2-T-C-X2-G), and the protein O-fucosyltransferase. Using TSR4 of the axonal guidance protein F-spondin as a model, we have investigated structural features that are important for the two forms of glycosylation. Residues that are part of the consensus sequence, or that are important for the 2-D and/or 3-D structure were replaced by Ala. Recombinant proteins expressed in HEK293T cells were purified and analysed by LC-MS. From the results we concluded that C-mannosylation in TSRs differs from that in RNase 2 in the following aspects: 1) the second Trp in the W-x-x-W sequence is important, but not essential, for C-mannosylation of the first Trp; 2) C-mannosylation of the second Trp requires the presence of the first one. It is presently unclear whether the two proteins are C-mannosylated by one and the same PCMT. A common feature of the C-mannosylation of TSR4 and RNase 2, appears to be the negative influence of the 2-D or 3-D structure. The results suggest that C-mannosylation has to occur before folding is complete. In contrast, the mutagenesis studies showed that O-fucosylation of TSR4 requires proper folding. In particular, disruption of the C-terminal part of the CWR stack of TSR4, or increasing the size of the loop between the first two strands, abolished O-fucosylation. The importance of the 3-D structure has also been found for the O-fucosylation of EGF repeats.

(129) **Synthesis of Proliferating Azido-LacNac Derivativesas Precursors of a Combinatorial Diverse LacNac Library for the Generation of Potent Galectin Binders**

Katja Michael, Qianli Wang and Adytia K. Sanki
Department of Chemistry, University of Hawaii, Honolulu, HI 96822, U.S.A..

Galectins are a family of 14 beta-galactoside binding endogenous lectins with conserved features in the carbohydrate recognition region. Certain poly-N-acetyl-lactosamine containing glycoconjugates, e.g., laminin, are among the strongest binding ligands for galectin. LacNac itself is also a galectin binder albeit with weak affinity of only 190 uM. Galectins are known to play key roles in cell growth regulation, cell adhesion, tumor progression, and metastasis, but their precise biological roles are not understood. Due to their participation in pathogenic processes there is a great interest in generating novel galectin binders as tools to study the galectins' glycobiology, and perhaps even to develop galectin blocking drugs. As demonstrated by a crystal struture of the galectin-3/LacNac complex,[1] OH-4' and OH-6' of Gal, and OH-3 of GlcNac are involved in direct electrostatic contacts with amino acids in the core binding region. The crystal structure also reveals that some of LacNac's hydroxyls (OH-3', OH-4', and OH-6' of Gal, and OH-6 of GlcNac) are involved in hydrogen

bonds to bound water molecules, while OH-2' of Gal and OH-1 of GlcNac don't show any contacts, suggesting that they are pointing away from the binding pocket. Therefore, OH-2' and OH-3' of Gal, and OH-1 and OH-6 of GlcNac are prime positions for derivatization without interfering with direct intermolecular hydrogen bonds important for molecular recognition. Generating a large structurally diverse LacNac library by parallel synthesis is not very practical, since each disaccharide derivative would be the result of a time-consuming and laborious multiple step synthesis. Here we demonstrate the synthesis of a few (< 10) highly biased LacNac library precursors, which will later proliferate into a structurally diverse LacNac library by chemical modification. Each of the precursors contains the _eta-galactosyl moiety as a basic recognition element, known to account for specificity in galectin binding. Furthermore, each precursor is functionalized with one or two azido groups, and with a fluorescent label, covering all possible positions of the LacNac scaffold that are not involved in binding to the galectin core binding region. The azido groups allow for the chemoselective reaction with a number of different small molecules with terminal alkynes generating triazole linkages via click chemistry.[2,3] LacNac derivatives with one azido function can react to linear LacNac ligands; LacNac derivatives with two azido functions can react with dialkynes to afford cyclic, conformationally constrained LacNac ligands. Thus, from our precursors a large structurally diverse library can be created and screened for galectin binders with novel structural features. References [1] J. Seetharaman, A. Kanigsberg, R. Slaaby, H. Leffler, S. Barondes, J. M. Rini, J. Biol. Chem. 1998, 273, 13047. [2] C. W. Torn¯e, C. Christensen, M. Meldal, J. Org. Chem. 2002, 67, 3057. [3] H. C. Kolb, M. G. Finn, K. B. Sharpless, Angew. Chem. Int. Ed. 2001, 40, 2004.

(130) **Studies on the Sialyltransferase that Forms the NeuAcα2-6GlcNAc Structure in Oligosaccharide Chains of N-linked Glycoproteins**

James C Jamieson and Erin M Szidonya
Department of Chemistry, University of Manitoba, Winnipeg, Canada.

Rat, human and bovine glycoproteins contain the structure NeuAcα2-6GlcNAc. The sialyltransferase that adds NeuAc is described as ST6GlcNAcI and has a specificity for addition to NeuAcα2-3Galβ1-3GlcNAc terminal trisaccharides on oligosaccharides of the N-linked type. We have used oligosaccharides of defined structure to act as acceptors for the enzyme, and more recently, we have developed macromolecular acceptors for the enzyme. Starting materials were fetuin and bovine α1-acid glycoprotein. Fetuin contains O- and N- linked chains. To remove the O-linked chains fetuin was treated with sodium borohydride in presence of cadmium acetate/EDTA which protects the N-linked chains while specifically cleaving the O-linked chains. NeuAc was then removed by neuraminidase or dilute acid treatment and Galβ1-4 was removed with *Streptococcus pneumoniae*. The Galβ1-3 chains that remained were recharged with ST3GalIII to produce the correct acceptor structure. At each step in the conversion process acceptors were checked for their ability to react with ST6GalI, ST3GalIII and ST3GalII. Bovine α1-acid glycoprotein does not contain O-linked chains so that borohydrate treatment was not necessary. Both acceptors were used in kinetic studies with rat liver Golgi as source of enzyme, at each stage checking that the correct product of the reaction was formed. Using liver from rats suffering from the acute phase response ST6GlcNAcI was found to be an acute phase reactant.

Supported by NSERC Canada.

(131) **An Assay Development for Ganglioside Sialyltransferases**

Cynthia Sun and Jens Sommer-Knudsen
Industrial Research Limited, PO Box 31-310, Lower Hutt, New Zealand.

Gangliosides are a class of cell membrane glycolipids that contain a core ceramide structure and an oligosaccharide chain with one or more sialic acid residues. They are involved in several important biological functions, such as brain development, cell-cell recognition and adhesion. Some gangliosides have been also described as markers for tumour cell growth. Ganglioside sialyltransferases are located in the golgi apparatus of the cell, where they transfer sialic acid to the cell-surface gangliosides. Assays for determining sialyltransferase activities have been dominated by radiometric methods, which use the radiolabelled donor substrate CMP-sialic acid. Other fluorometric assays, with higher sensitivity than the radiometric assays, have been developed since 1990, but none of the fluorescent labelled substrates in these methods are commercially available for routine laboratory applications. This work presents a new method for the determination of ganglioside sialyltransferase SAT IV (EC 2.4.99.2), which utilizes the commercial available GM1-BODIPY as the acceptor substrate. In this assay, HPLC coupled with a fluorescence detector is used to analyse the substrate changes in the enzymatic reaction, which allows the analysis to be performed in a quick and automated fashion. This method achieved a good linearity for the concentration of GM1-BODIPY with a detection limit of 0.15 pmol, which is significantly lower than that of most radiometric methods. Therefore, the consumption of both substrates is much less than that in radiometric methods.

(132) **Structural Characterization of the Sialic Acid Synthase NeuB from *Neisseria meningitides* in the Presence of Mn^{2+} and Phosphoenolpyruvate**

Jason Gunawan[1], Andrew L. Lovering[1], Michel Gilbert[2], Warren W. Wakarchuk[2] and Natalie C.J. Strynadka[1]
[1] University of British Columbia, Department of Biochemistry and Molecular Biology, 2146 Health Sciences Mall, Vancouver, British Columbia, V6T1Z3, Canada, [2] Institute for Biological Science, National Research Council of Canada, 100 Sussex Drive, Ottawa, Ontario, Canada, K1A0R6.

Sialic acids are a family of high carbon sugars that play a vital role in variety of biological functions, such as development, recognition, and cell adhesion, in viruses, mammalian cells, and microbial organisms. These nine carbon 3-deoxy-2-keto sugars are commonly found as a series of repeating units on the terminal ends of secreted and cell surface glycoproteins and glycolipids on the aforementioned organisms. Several species of pathogenic bacteria, such as Escherichia coli, and Neisseria meningitidis, produce sialylated capsular polysaccharides in order to avert host defenses. In addition to mimicking human gangliosides, sialylated polysaccharides block targets commonly recognized by innate immunity responses. A variety of pathways involved in the synthesis and degradation of sialic acids in bacteria and eukaryotes have been identified; however, little is known about the structural and mechanistic properties of the enzymes involved in their creation. N.meningitidis, the causative agent of meningitis, encodes a sialic acid synthase, referred to as NeuB, which directly converts phosphoenolpyruvate (PEP) and N-acetylmannosamine (ManNAc) into N-acetylneuraminic acid (NeuNAc, or sialic acid). It is the goal of this project to understand the catalytic mechanism of sialic acid synthesis in terms of the structural characteristics of NeuB. Purification of recombinant NeuB involving several chromatographic steps was carried out to high levels of purity. Native, selenomethionine, and mercury incorporated orthorhombic crystals of NeuB, in the space group $P2_12_12$, with unit cell dimensions a = 58.37 ≈,b = 76.16 ≈, c = 77.74 ≈ were obtained. Crystals of NeuB were solved through SAD phasing from incorporated selenomethionines. NeuB exhibits a domain swapped dimer architecture consisting of a $(\Diamond/\langle)_8$ barrel (TIM barrel) type fold at the N-terminal end and an antifreeze - like domain at the C-terminal end of the enzyme. Typical of other TIM barrel proteins, the active site of NeuB is located in a cavity at the C-terminal end of the TIM barrel. However, the positioning of the swapped antifreeze-like domain from the adjacent polypeptide suggests a possible catalytic role, a structural property not seen in other enzymes that catalyze aldol-type condensations.Site-directed mutagenesis of conserved residues within the active site will be carried out in order to understand their effects kinetically and crystallographically. Due to the prevalence of meningitis in the world today, structural information of NeuB gathered through this project will hopefully lead to the development of novel therapeutic inhibitors against this debilitating ailment.

(133) **The Dendritic Cell Expressed C-Type Lectin Mgl Specifically Interacts with Terminal Galnac Residues**

Sandra J. van Vliet[1], Eirikur Saeland[1], Tatsuro Irimura[2], Teunis B.H. Geijtenbeek[1], Irma van Die[1] and Yvette van Kooyk[1]
[1] Dept. of Molecular Cell Biology & Immunology, VU medical Center, Amsterdam, the Netherlands, [2] Dept. of Cancer Biology & Molecular Immunology, University of Tokyo, Tokyo, Japan.

The C-type lectin MGL is expressed by immature dendritic cells (DC) and macrophages in the human skin. The MGL protein contains one Carbohydrate Recognition Domain (CRD). Little is known about the actual carbohydrate specificity of MGL for self-glycoproteins or pathogens and how this recognition influences the immune system. We generated a MGL-Fc chimeric protein to facilitate efficient screening of potential membrane and soluble ligands or carbohydrates. Using a glycan micro-array system we identified a terminal GalNAc-specifity for MGL. Based on the results from the array, we identified helminth specific glycans in the soluble egg antigens of *Schistosoma mansoni* that interact with MGL. Furthermore,

terminal GalNAc residues are associated with tumor pathogenesis. Indeed, tumorcells, either from T cell, melanoma or adenocarcinoma origin, were bound with high affinity by MGL. We identified MUC1 as the MGL ligand in the adenocarcinoma cell line ZR75-1. MGL recognition of MUC1 was specific for tumor-derived MUC1 as normal MUC1 expressed by mature DC did not interact with MGL. These results implicate a role for MGL in the pattern recognition of helminths and tumors by DC.

(134) **A Novel 'Cased' Fluorogenic Substrate for O-GlcNAcase and Its Variant**

Eun Ju Kim and John A. Hanover
Laboratory of Cell Biochemistry and Biology, NIDDK, National Institutes of Health, MD 20892.

The O-GlcNAc specific β-N-acetylglucosaminidase (O-GlcNAcase) is thought to be involved in neurological disorders, diabetes and cancer. Growing interest in O-GlcNAcase stimulated the need for a highly sensitive enzymatic assay method. Since one of the simplest and most sensitive methods involves fluorescence, florescein was selected as fluorogenic substance and a novel °ÆCased°Ø fluorogenic substrate, fluorescein di-β-D-acetylglucosamine, was synthesized. Its synthesis involves modified Koenigs-Knorr reaction by which N-acetyl-D-glucosaminyl group is conjugated into fluorescein with β-linkage. Kinetic analysis using purified O-GlcNAcase and its splice variant (*v*-O-GlcNAcase), a protein lacking the C-terminal third of the full-length O-GlcNAcase, expressed in *Escherichia coli* suggests that this fluorogenic compound is a much more efficient substrate (Km = 38 μM) than the conventional substrate, para-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (PNP-GlcNAc, Km = 1.1 mM) for O-GlcNAcase. The variant O-GlcNAcase exhibited a Km of 655 μM with respect to the novel fluorogenic substate. This isoform was not thought to have O-GlcNAcase activity based on *in vitro* studies with PNP-GlcNAc examined by Hart et al. (*J. Biol. Chem.* 2002, **277**, 1755). However, both enzymes reduced O-GlcNAc level of proteins extracted from HeLa and HT-29 cells *in vitro*, indicating that the splice variant has also O-GlcNAcase activity as shown in enzymatic assay with this new substrate, even though the activity found in *v*-O-GlcNAcase is lower than that possessed in the full-length O-GlcNAcase. On the other hand, fluorescein di-β-D-acetylgalactosamine is not cleaved by these enzymes as reported previously that the O-GlcNAcase has substrate specificity toward O-GlcNAc and not O-GalNAc. The novel fluorogenic substrate is found to be a highly sensitive and useful tool for studying both enzymes. In addition, a high-throughput analysis is possible using a multiwell plate format with this new substrate.

It is still unknown what the role of this splice variant is in a cellular context and much of O-GlcNAcase remains unexplored. One area of the exploration is regulation of these enzymes by either overexpression or inhibition. O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenylcarbamate (PUGNAG) is known as a potent inhibitor of the O-GlcNAcase. In order to find inhibitors which selectively block activity of one of the enzymes so as to further understand the enzymatic mechanism as well as the functional relationship of O-GlcNAcase proteins with their structure, our effort has been made to prepare analogues of PUGNAc and GlcNAc. Several analogues of PUGNAc and GlcNAc have been prepared and analyzed using the new fluorogenic substrate.

(135) **Carbohydrate-Binding Specificity of a Fucose-specific Lectin from *Aspergillus oryzae***

Kengo Matsumura[1], Katsuya Higashida[1], Hiroki Ishida[1], Yoji Hata[1], Akitsugu Kawato[1], Yasuhisa Abe[1], Michiko Kato[2], Mitsuyoshi Ueda[2] and Kenji Yamamoto[3]
[1] Research Institute, Gekkeikan Sake Co. Ltd, [2] Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, [3] Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University.

ÅyIntroductionÅz The filamentous fungus, *A. oryzae*, is one of the most important fungi in the Japanese fermentation industries such as sake, shoyu and miso manufacturing. We found a novel fucose-specific lectin (AOL) in the iron-deficient culture of *A. oryzae* and isolated the corresponding gene, *fleA*.[1)] The carbohydrate-binding specificity of the AOL was investigated using surface plasmon resonance analysis (BIACORE) for comparison with that of *Aleuria aurantia* fucose-specific lectin (AAL) known as highly specific for αFuc1–6 linked oligosaccharides. ÅyMethodsÅz To clarify the function of the AOL, the *fleA* was overexpressed in *A. oryzae*, resulting for 1000-times enhancement of AOL production. The AOL was purified to such an extent that it resulted in agglutination of rabbit red blood cell at 2.0 μg-Lectin/ml in PBS (pH7.2). We made a comparison between the carbohydrate-binding specificity of the AOL and that of the AAL using BIACORE with fucosyl oligosaccharides, because the AOL shows approximately 26% similarity at amino acid level with the AAL and the specificity of the AOL for L-fucose is equivalent to the AAL. In addition the three dimensional structure of the AOL is similar to the AAL: homo-dimer with the subunit molecular weight of 35,000 and 33,383, respectively. We investigated the interaction of six pyridylamino (PA)-sugar chains of different fucosyl oligosaccharides with the AOL and the AAL immobilized to a CM5 sensor chip by amine coupling with 79.7 and 101 fmol/mm^2, respectively. PA-sugar chains solutions (1 nmol/ml in 10 mM HEPES pH 7.4, containing 0.15 M NaCl, 100 μl) were injected and the amount of bound PA-sugar chains was determined as the difference in resonance units (RU) before and after the injection start.

ÅyResultsÅz BIAcore analysis showed that AOL has the strongest preference for αFuc1–6 linkage among 1–2, 1–3, 1–4 and 1–6 linked oligosaccharides in a similar manner as AAL, while AOL bound more weakly to the second preferableαFuc1–2 linked oligosaccharides of them than AAL. These data showed that AOL is more specific for the αFuc1–6 linked oligosaccharides than AAL.

ÅyConclusionsÅz AOL is an important probe to αFuc1–6 linked carbohydrates on cell surface.

[1)] Molecular cloning and overexpression of *fleA* gene encoding a fucose-specific lectin of *Aspergillus oryzae*. *Biosci. Biotechnol, Biochem.*, 66(5), 1002-1008 (2002) H. Ishida, T. Moritani, Y. Hata, A. Kawato, K. Suginami, Y. Abe, S. Imayasu

(136) **Immunomodulatory Effect of Mucins Produced by Epithelial Cancer Cells**

Ippei Sugihara, Munetoyo Toda, Mizue Inoue and Hiroshi Nakada
Dept. Biotechnology, Kyoto Sangyo Univ.

Mucins produced by cancer cells are secreted into tissues and/or sera of cancer patients. Although high levels of serum mucins have been shown to correlate with poor prognosis in human cancer patients, little is known regarding the biological significance of mucins. From other point view, several epidemiological and chemical studies have shown that nonsteroid antiinflammatory drugs, which are known to inhibit cyclooxygenase (COX), can decrease risk of cancer development. Two forms of COX, i.e. COX-1 and COX-2, which are constitutive and inductive enzyme, respectively, have been identified. Expression of COX-2 and the production of its major product, prostaglandin E2 (PGE2), are elevated in various human cancers. It has been reported that PGE2 plays various roles as a biological mediator. Recently we have demonstrated that mucins isolated from human colon cancer cells could activate monocytes/macrophages through a scavenger receptor (SCR), resulting in induction of COX-2 and subsequent overproduction of PGE2. In the present study, we investigated immunomodulatory effect of mucins through PGE2 produced by macrophages. First, we examined the effect of mucins on production of PGE2 using mouse peritoneal macrophages. Epiglycanin, which is a mucin-like glycoprotein and produced by a mouse mammary adenocarcinoma, TA3-Ha, was purified from the extract of TA3-Ha tumor tissues. Mouse peritoneal macrophages were prepared from a thioglycolate-treated mouse using anti-CD11b Ab conjugated magnetic beads, cultured in the presence of epiglycanin or bovine submaxillary mucin (BSM), and then secreted PGE2 was estimated by ELISA. Production of PGE2 was enhanced by incubation with these mucins in a dose dependent manner. To determine whether this enhancement was associated with up-regulation of COX-2, COX-2 mRNA level was determined after treatment with various concentrations of mucins. A small amount of these mucins could induce COX-2 mRNA, indicating a COX-2 mediated pathway. Next, we examined the effect of mucins on production of antibody against sheep red blood cells (SRBC) using plaque method. Mouse spleen cells were cultured with SRBC in the presence or absence of mucin for five days and then incubated with fresh SRBC in 0.5 % agarose. After addition of fresh guinea pig serum, plaques were counted. The number of plaques increased about three fold by treatment with mucin. Next, we examined the effect of mucins on production of macrophage-derived IL-12 and T cell-derived IFN-gamma. Mouse peritoneal macrophages were cultured in the presence or absence of mucin for 20 h and then the cells were treated with LPS for 18 h. IL-12 p70 secreted into the culture medium was assayed by ELISA. Treatment with mucin decreased the production of IL-12 p70 significantly. After treatment of macrophages with mucin as described above, CD4$^+$ T cells were added and cultured in the presence of LPS for 18 h. IFN-gamma secreted into the

culture medium was assayed by ELISA. Production of T cell-derived IFN-gamma was also strongly suppressed by treatment with mucin. Taken together, there results suggest that mucins enhance the production of PGE2 from macrophages, leading to suppress Th1-related immune responses and augment Th2-related immune responses.

(137) **Heparan Sulfate Proteoglycans Interact with Neurocan and Promote Neurite Outgrowth from Cerebellar Granule Cells**

Yuki Hosoki[1], Kaoru Akira[1], Munetoyo Toda[1], Mizue Inoue[1], Shinji Fushiki[2], Atsuhiko Oohira[3], Minoru Okayama[1] and Hiroshi Nakada[1]

[1] Dept. biotechnology, Kyoto Sangyo Univ, [2] Department of Pathology and Applied Neurobiology, Kyoto Prefectural University of Medicine Graduate School of Medical Science, [3] Institute for Developmental Research, Aichi Human Service Center.

It is known that syndecan-3 is a membrane bound heparan sulfate proteoglycans (HSPG) and expressed on the growing axonal surface during brain development. First, we performed a ligand overlay assay with biotinylated soluble syndecan-3 to detect binding proteins for syndecan-3. One of the binding proteins was purified from mouse brains and analyzed biochemically. Treatment of the binding protein with chondroitinase ABC produced three molecular species exhibiting molecular weights of 260 kDa, 150 kDa and 130 kDa on SDS-PAGE. Sequence analysis of 260 kDa and 130 kDa components was revealed to be the same amino terminal sequence, i.e. DQPTQDTTA, which coincided with that of neurocan, indicating that these core proteins correspond to the whole molecule and the N-terminal fragment of neurocan, respectively. The N-terminal amino acid sequence of the 150 kDa core protein was LRAPKLWLP, which coincided with the N-terminal sequence of the C-terminal fragment of neurocan. Next, we asked whether neurocan could bind to other HSPGs or not. We tried to isolate neurocan binding protein using neurocan-Sepharose. Eluates from neurocan-Sepharose was subjected to SDS-PAGE before or after treatment with heparitinase I followed by immunoblotting. The major core band with 62 kDa was revealed to be glypican-1 by sequence analysis and immunoreactivity to anti-glypican-1 antibodies. It has been reported that these HSPGs interact with some extracellular matrix components and these interactions are probably involved in some biological functions such as cell adhesion, cell migration and neurite outgrowth. Thus, we investigated binding property between HSPGs and neurocan and its biological significance. The binding of these HSPGs to neurocan was prevented by treatment of these HSPGs with heparitinases I and II, but not by treatment of neurocan with chondroitinase ABC. Scatchard plot analysis indicated that neurocan has two binding sites for these HSPGs with different affinities. It is known that neurocan in the rodent brain is proteolytically processed with age into N- and C-terminal fragments. When a mixture of whole neurocan and N- and C-terminal fragments prepared from neonatal mouse brains or recombinant N- and C-terminal fragments were applied to a heparin column, the whole molecule and both the N- and C-terminal fragments bound to heparin. Centrifugation cell adhesion assay indicated that both the N- and C-terminal neurocan fragments could interact with these HSPGs expressed on the cell surface. To examine the biological significance of HSPGs-neurocan interaction, cerebellar granule cells expressing these HSPGs were cultured on the recombinant neurocan substrate. Prominent increase of neurite outgrowth was observed on the wells coated with the C-terminal neurocan fragment, but not with the N-terminal one. Neurite outgrowth-promoting activity was inhibited by pre-treatment of neurocan substrate with heparin or addition of heparitinase I into culture medium. These results suggest that HSPGs such as syndecan-3 and glypican-1 serve as the cell surface receptor of neurocan, and that the interaction of these HSPGs with neurocan through its C-terminal domain is involved in the promotion of neurite outgrowth*. * K. Akita et al. Biochem. J., in press

(138) **Synthesis and Property of Carbosilane Dendrimers Functionalizing Peripheral Mannose Moieties**

Tomonori Mori[1,2], Ken Hatano[1], Koji Matsuoka[1], Yasuaki Esumi[3], Eric J. Toone[4] and Daiyo Terunuma[1]

[1] Department of Functional Materials Science, Saitama University, [2] Japan Association for the Advancement of Medical Equipment, [3] The Institute of Physical and Chemical Research (RIKEN), [4] Department of Chemistry, Duke University.

Mannose is one of the important component of *N*-glycans. In particular, the *N*-glycan, highly accumulating mannose, is called high mannose type. Clustering peripheral mannose on carbosilane dendrimer would be able to mimic the high mannose type *N*-glycan and the cell surface of HIV or bacteria. In our ongoing synthetic study of neoglycoconjugates (artificial glycoconjugetes), synthetic assembly of carbohydrate moieties using carbosilane dendrimers have been achieved using lactose, globotriaose, sialyllactose, and so on. So we are described herein the preparation and characterization of a series of carbosilane dendrimers carrying mannose and its oligomeric derivatives, which have α-glycoside bond of the aglycon moiety. Mannose monosaccharide derivative, 1-*O*-(3'-acetylthiopropyl)-D-mannopyranose (Man), was synthesized by three steps reactions from D-mannose; acetylation, 1-allylation, and then thioacetylation of olefin moiety. Mannose disaccharide derivative, 1-*O*-(3'-acetylthiopropyl)-2,4,6-tri-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-D-mannopyranosyl) D-mannopyranose (Man-1,3-Man), was synthesized starting from D-mannose. Bromo 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose (Man-Br) was prepared from D-mannose. This is the key compound for the disaccharide synthesis because Man-Br itself was used as a glycosyl donor and 4,6-*O*-benzylidene-1,2-*O*-ethylidene-D-mannopyranose was synthesized by three steps reactions from Man-Br and used as a glycosyl acceptor of the mannose disaccharide derivative. Protecting groups of this disaccharide derivative were exchanged for acetyl group. Man-1,3-Man was synthesized after 1-allylation and following thioacetylation of olefin moiety. Mannose derivatives were introduced in carbosilane dendrimers of which generation was the zero and first. The zero generation carbosilane dendrimer scaffolds, which consist of Fan(0)3 type (three-branched) and Ball(0)4 type (four-branched), were synthesized using triallylphenylsilane and tetraallylsilane by following three steps reactions, hydroxylation, mesylation, and bromination. On the other hand, the first generation carbosilane dendrimer scaffold was prepared allylation of dichlorodimethylsilane, next hydrosilylation having the first generation skelton, following reactions were the same as the zero generation carbosilane dendrimer scaffolds. This type of dendrimer was named Dumbbell(1)6 type (six-branched).

Introduction of Man and Man-1,3-Man to carbosilane dendrimer scaffolds were done on the condition of Zemplen's manner, that is, using sodium methoxide/ methanol and *N*,*N*-dimethylformamide. As a result, six types of carbosilane dendrimers were functionalized by acetyl-protected derivatives of mannose or mannose disaccharide (Man-1,3-Man). Yields of the introduction of mannose monosaccharide type carbosilane dendrimers were 62-76 %, and those of Man-1,3-Man were 30-35 %. Deprotected products were synthesized by deacetylation using sodium methoxide/ methanol, then saponification to afford corresponding carbosilane dendrimers having peripheral mannose and mannose disaccharide, and purificated by gel filtration. All six types of carbosilane dendrimers functionalized peripheral mannose moieties were synthesized and characterized by the measurements of ^{1}H and ^{13}C NMR, and high resolution mass spectrometry. NMR data showed that the α-glycoside bond between the anomeric position of mannose and the aglycon moiety is kept after the formation of carbosilane dendrimer and deacetylation. Isothermal titration calorimetry (ITC) was done for assuming binding assay between carbosilane dendrimer and concanavalin A (Con A). It was found that carbosilane dendrimers were binding to Con A more than free mannose (Man-OMe) and mannose disaccharide (Man-1,3-Man-OMe).

(139) **An Endogenous Heparin-binding Growth Factor, Pleiotrophin, Mediates Neuritogenic Activity of Embryonic Pig Brain-derived Chondroitin Sulfate/Dermatan Sulfate Hybrid Chains Toward Mouse Hippocampal Neurons in Culture**

Xingfeng Bao[1], Tadahisa Mikami[1], Shuhei Yamada[1], Takashi Muramatsu[2,3] and Kazuyuki Sugahara[1]

[1] Department of Biochemistry, Kobe Pharmaceutical Univeristy, Kobe, Japan, [2] Department of Health Science, Faculty of Psychological and Physical Sciences, Aichi Gakuin University, Nisshin, Japan, [3] Department of Biochemistry, Nagoya University School of Medicine, Nagoya, Japan.

Chondroitin sulfate/dermatan sulfate (CS/DS) chains, major heterogenous polysaccharides of the extracellular matrix in the central nervous system, play important roles in neuronal cell adhesion, migration and neurite formation (1,2). CS/DS hybrid chains purified from embryonic pig brains (E-CS/DS) bind various growth factors and promote neurite outgrowth toward cultured embryonic mouse hippocampal neurons (3). However, the mechanism underlying the neuritogenic activity of the E-CS/DS chains is poorly understood. Here, we show that pleiotrophin, a heparin-binding growth factor, is the predominant binding partner for E-CS/DS in the membrane-associated protein fraction of neonatal rat brain, and is produced by the hippocampal cell culture system. Subfractions of E-CS/DS, separated using a pleiotrophin-immobilized affinity column, exhibited distinct effects

on neurite outgrowth. Bound fractions (12%) displayed neuritogenic activities, while the unbound fraction did not. The low-affinity bound fraction (10%) gave dendritic morphology, whereas the high-affinity bound fraction (2%) gave axonal morphology. These observations suggested that endogenous PTN mediated the neurite outgrowth-promoting activities of the E-CS/DS in the in vitro assay system. Interaction analysis demonstrated that CS-B strongly inhibited binding of pleiotrophin to the E-CS/DS (IC_{50} = 0.24 μg/ml). CS-C inhibited moderately (IC_{50} = 1.47 μg/ml), but CS-A weakly (IC_{50} > 100 μg/ml). Disaccharide composition analysis of the E-CS/DS affinity fractions revealed a gradual increase in the proportion of HexUAα1-3GalNAc(4S) with the increasing affinity. HexUA(2S)α1-3GalNAc(6S) and HexUAα1-3GalNAc(4S,6S) were the richest in the low-affinity and high-affinity fractions, respectively. Furthermore, analysis of chondroitinase B digests of these affinity fractions indicated that iduronic acid residues were more sparsely distributed along the CS/DS chains in the bound fractions than in the unbound fraction. Taken together, these findings suggest that CS subpopulations with distinct sequences in mammalian brain play different roles in neurogenesis partially through regulating the functions of growth factors. The detailed binding sequences in the E-CS/DS for pleiotrophin remain to be characterized. 1) Bandtlow CE, and Zimmermann DR (2000) *Physiol. Rev.* 80, 1267-1290. 2) Sugahara K, Mikami T, Uyama T, Mizuguchi S, Nomura K, and Kitagawa H (2003) *Curr. Opin. Struct. Biol.* 13: 612-620. 3) Bao X, Nishimura S, Mikami T, Yamada S, Itoh N, and Sugahara K (2004) *J. Biol. Chem.* 279: 9765-9776.

(140) **A Novel Lectin (*Morus nigra*) from Mulberry (Morus nigra) Bark Recognizes Oligomannosyl Residues in *N*-Glycans**

AlbertM. Wu[1], June H. Wu[2], Tanuja Singh[1], Kang-Chuang Chu[1], Willy J. Peumans[3], Pierre Rouge[4] and Els J.M. Van Damme[3]

[1] Glyco-Immunochemistry Research Laboratory, Institute of Molecular and Cellular Biology, [2] Department of Microbiology and Immunology, College of Medicine,Chang-Gung University, Kweishan, Taoyuan, 333, Taiwan., [3] Department of Molecular Biotechnology, Faculty of Agricultural and Applied Biological Sciences, Ghent University, Coupure Links, Gent, Belgium., [4] Surfaces Cellulaires et Signalisation chez les Vegetaux, UMR-CNRS 5546, Pole de Biotechnologie vegetale, Castanet Tolosan, France.

Morniga M is a jacalin-related and mannose-specific lectin isolated from the bark of the mulberry (*Morus nigra*). In order to understand the function and application of this novel lectin, the binding property of Morniga M was studied in detail using an enzyme-linked lectinosorbent assay (ELLSA) and lectin-glycan inhibition assay with extended glycan/ligand collection. From the results, it was found that the di-, tri-, and oligomannosyl structural units of *N*-glycans such as those of the bovine α_1 acid gp and lactoferrin, were the most-active glycoproteins, but not the *O*-glycans or polysaccharides including mannan from yeast. The binding affinity of Morniga M for ligands can be ranked in decreasing order as follows: glycoproteins carrying multiple *N*-glycans with oligomannosyl residues >> *N*-glycopeptide with a single trimannosyl core > Tri-Man oligomer (Manα1-6(Manα1-3)Man), Penta-Man oligomer (Manα1-6(Manα1-3)Manα1-6(Manα1-3) Man) > Man α1-2, 3 or 6 Man > Man > GlcNAc, Glc >> LFuc, Gal, GalNAc (inactive), demonstrating the unique specificity of this lectin that may not only assist in our understanding of cell surface carbohydrate ligand-lectin recognition, but also provide informative guidelines for the application of this structural probe in biotechnological and clinical regimens, especially in the detection and purification of *N*-linked glycans.

(141) **Shedding Light on the Carbohydrate-mediated Self-recognition of Marine Sponge Cells**

Adriana Carvalho de Souza, Koen M. Halkes, Johannes F.G. Vliegenthart and Johannis P. Kamerling

Bijvoet Center, Department of Bio-Organic Chemistry, Section of Glycoscience and Biocatalysis, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.

The species-specific cell adhesion of marine sponges involves proteoglycan-like macromolecular complexes otherwise known as aggregation factors (Fernandez-Busquets and Burger, 2003). In the case of *Microciona prolifera*, in a Ca^{2+}-independent process, a 6 kDa glycan (g-6) located on aggregation factor arms adheres to cell surface receptors, while a 200 kDa glycan (g-200) protruding from the aggregation factor central ring promotes cell adhesion via a Ca^{2+}-dependent self-association process. One of the carbohydrate epitopes involved in the g-200 self-association is the sulfated disaccharide Glc*p*NAc3*S*(β1-3)Fuc*p* (Spillmann *et al.*, 1995). In order to try to mimic the self-recognition on the disaccharide molecular level, β-D-Glc*p*NAc3*S*-(1-3)-α-L-Fuc*p*-(1-$OCH_2CH{=}CH_2$) [GF] was synthesized, and conjugated in several ways for SPR, TEM and AFM studies in the presence and absence of 10 mM $CaCl_2$. SPR studies using GF-BSA neoglycoproteins (GF elongated with cysteamine, then linked via diethyl squarate) strongly supported the existence of true carbohydrate self-recognition, in the presence of Ca^{2+}-ions, as a major force in the aggregation phenomenon (Haseley *et al.*, 2001). In additional TEM studies, using gold nanoparticles coated with GF via an extended thiol spacer, only in the presence of Ca^{2+}-ions aggregation was observed (clusters up to 100 nm diameter). TEM studies of gold glyconanoparticles decorated with synthetic carbohydrates related to the sulfated disaccharide GF (β-D-Glc*p*NAc3*S* and α-L-Fuc*p* alone; structural changes on the disaccharide level: α-L-Fuc*p* replaced by β-L-Fuc*p*, β-D-Glc*p*NAc3*S* replaced by β-D-Glc*p*NAc, β-D-Glc*p*NAc3*S* replaced by β-D-Glc*p*3*S*, and α-L-Fuc*p* replaced by α-L-Gal*p*) demonstrated that (a) both units of GF are necessary for self-recognition, (b) the disaccharide containing α-Fuc (GF) has a much stronger self-recognition than the disaccharide with β-Fuc (clusters up to 15 nm diameter), (c) the other disaccharide variants do not show any aggregation in the presence or absence of Ca^{2+}-ions, which means that the hydrophobic methyl group of α-Fuc has an irreplaceable function in the self-recognition, and that the sulfate and *N*-acetyl groups are involved in the carbohydrate self-recognition, possibly, through the coordination of Ca^{2+}-ions. Finally, to measure the adhesion forces by AFM, flat gold-surface samples and gold cantilevers were functionalized with self-assembling monolayers of a thiol-spacer-containing sulfated disaccharide (extended GF). It turned out that in the presence of Ca^{2+}-ions, the force required to separate the tip and the sample both coated with disaccharide was found to be quantized in integer multiples of 30±6 pN. No binding was observed between the monolayers in the absence of Ca^{2+}-ions. A possible mechanism will be proposed.

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(142) **Selection of Ganglioside GM1-binding Peptides with a Helix-loop-helix Scaffold**

Mie Iida[1], Teruhiko Matsubara[1], Takeshi Tsumuraya[2], Ikuo Fujii[2] and Toshinori Sato[1]

[1] Department of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan, [2] Research Institute for Advanced Science and Technology, Osaka Prefecture University, 1-2 Gakuen-cho, Sakai, Osaka 599-8570, Japan.

Cell surface is coated with various sugar chains of glycoproteins, glycolipids and proteoglycan which are concerned in disease and receptor for virus or toxin. The peptide sequences which bind to carbohydrate chain might be applied to tools for the investigation of the biological functions of glycoconjugates, and the therapy for carbohydrate-related diseases.

The ganglioside GM1(Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1'ceramide) is known as the receptor for cholera toxin B subunit(CTB). In this study, amino acid sequence of GM1-binding peptide was identified by phage display method. Combinatorial peptide libraries are useful sources for screening bioactive ligands that bind to receptors and enzymes. However, the small peptides generally possesses considerable conformational flexibility, so the entropic loss for binding to target molecules are high. Therefore, we designed a conformationally restricted peptide library having a helix-loop-helix scaffold. Five resides of C-terminal helix were randomized to give a library of helix-loop-helix peptides. A random library of helix-loop-helix peptides was displayed on the major coat protein of M13 filamentous phage. We carried out an affinity selection with a GM1 monolayer prepared at an air-water interface. Hydrophilic carbohydrate portion of glycolipid at the monolayer are exposed to a water phase. Therefore, phages will interact only with the carbohydrate portion, but not with the hydrophobic lipid of ceramide. After 7-9 rounds of the selection, the DNA sequence of 60 isolated phages showed that 29 individual clones were selected. Binding affinity of the selected phage clones for the GM1 monolayer were determined by ELISA, only two phage clones bound to GM1. The two peptides were chemically synthesized and the binding affinity of the peptides for GM1 was evaluated by surface plasmon resonance method. The synthetic peptides had high affinity for GM1 with dissociation constant of 1Å~10^{-6} M, whereas they did not bind to glucosylceramide. In the presence of the peptides, the binding of CTB to

GM1 was inhibited with IC_{50} value of 2.7Å~10^{-6} M. These results indicated that the peptides recognize a sugar moiety of GM1. A circular dichroism analysis in water indicated that the synthetic peptide took a helical conformation as we expected. A half-peptide, which cannot take a stable helical conformation, had low affinity for GM1. This result indicated that the binding affinity of peptide was enhanced by the helix-loop-helix structure. We proved that the secondary structure of peptide was important for binding to sugar. Furthemore, we prepared several mutants replaced the selected amino acid with alanine. The binding affinity of all mutant peptides for GM1 was decreased. It was indicated that the sugar-peptide interaction was mediated by the selected amino acid.

(143) **Recombinant α-dystroglycan Produced in CHO Cells is Heavily Glycosylated Independent of β-dystroglycan Expression, and Binds to Recombinant Agrin Domains in a Ca++- and Sialylation-independent Fashion**

Qiang Qu and Frances Smith
University of Massachusetts Medical School, Shriver Center for Mental Retardation, 200 Trapelo Road, Waltham, MA, 02452.

Dystroglycan (DG) is a component of the dystrophin-glycoprotein complex that is post-translationally cleaved to produce α- and β- subunits (α-DG and β-DG) that remain non-covalently associated. α-DG is heavily modified with O-mannosyl-linked carbohydrate chains, and genetic defects resulting in abnormal glycosylation of α-DG can lead to abnormalities of both muscle and brain. CHO cell lines that possess well-characterized defects in defined cellular glycosylation pathways, offer an interesting system for investigating the glycans and protein domains required for α-DG interactions with its various ligands. CHO cell lines are demonstrated to express the genes that have been associated with defects in α-DG glycosylation (POMT1, POMGnT1, fukutin, and Large) and glycosylation of recombinant α-DG is shown to be independent of β-DG or membrane association. ELISA assays were used to investigate α-DG-ligand interactions, and show that recombinant α-DG produced in CHO cells exhibits preferential binding to M isoforms rather than to N isoforms of agrin, as previously reported for α-DG isolated from muscle. Agrin binding affinity to α-DG was comparable for α-DG produced in GAG-deficient cells (CHO618), complex N-glycan deficient cells (CHOP1), and sialylated glycan deficient cells (CHOP2/1 cells), suggesting that these types of glycans are not required for agrin binding. Deletion mutants of α-DG mapped agrin binding to the C-terminal half of the α-DG molecule. α-DG isolated from CHO cells bound agrin at lower affinity than that isolated from muscle, and was independent of Ca++ . These results indicate that the agrin-binding properties of α-DG produced by these CHO cell lines differ significantly from those present in α-DG isolated from muscle, probably due to differences in glycan content.

(144) **Pancreatic Proteinases Show Novel Carbohydrate-binding Activities and Bind to Glycoreceptors in the Intestinal Brush Border Membrane**

Hiroko Takekawa, Chieko Ina, Yuki Ihara and Haruko Ogawa
Graduate School of Humanities and Sciences, Ochanomizu University, Tokyo 112-8610 Japan.

Introduction Trypsin is a major pancreatic serine protease specific to peptide bonds on the carboxyl side of positively charged lysine and arginine. Trypsinogen is a proenzyme of trypsin and is proteolytically activated after secretion from the pancreas into the duodenum. In the duodenum, trypsin activates other proteinase zymogens, and they cooperatively break down dietary proteins. Porcine pancreatic α-amylase (PPA)[1] has been found to have carbohydrate-binding activity toward *N*-linked oligosaccharides. Because the activity is present in α-amylase from pancreas but not from saliva, plants and fungi, we investigated carbohydrate-binding activities of principal pancreatic enzymes to examine the biological significance of the carbohydrate-specific interaction. In this study, trypsin, trypsinogen and chymotrypsin from mammalian pancreas were found to exhibit carbohydrate-binding activities and to interact sugar-specifically with their intestinal glycoreceptors to enhance the activity. Methods and Results A series of biotinylated glycoprotein probes and their deglycosylated derivatives were prepared. Interactions between the glycoprotein probes and porcine or bovine pancreatic trypsin (PPT, BPT), bovine trypsinogen (BPTG), and chymotrypsin (BPC) were studied by ELISA. Both PPT and BPT bound to bovine thyroglobulin, fetuin, and ovomucoid, but not to bovine submaxillary mucin (BSM) at pH 7.5, which is the pH in the duodenum. BPC bound to bovine and porcine thyroglobulin but not to BSM. On the other hand, BPTG bound not only to glycoproteins possessing *N*-linked glycans but also to BSM, which has only *O*-linked mucin-type glycans at pH 5.5, the pH of zymogen granules. Binding studies with sugar-biotinylated polyacrylamide probes (sugar-BP probes) indicated that both trypsins have affinity to α-Man-, α-Man-6-phosphate-, and α2-6-NeuAc-BP-probes, in that order, and that BPC bound to α-D-Gal-, β-D-Glc-, and α-D-Man-BP-probes at pH 7.5, while BPTG bound to α-Man-, α-Man-6-phosphate-, and α-GalNAc-BP-probes at pH 5.5. No carbohydrate-binding activity was inhibited by EDTA, suggesting that the carbohydrate binding is independent of Ca^{2+}. To search for the endogenous glycoreceptor for trypsins, the brush border membrane (BBM) was extracted from porcine duodenum and solubilized with detergent. The BBM fraction was separated by SDS-PAGE and transferred to a PVDF membrane. Biotinylated PPT bound to several protein bands of the BBM on the PVDF membrane, which were revealed to possess *N*-linked glycans by reactivity with lectins, SNA, LCA, or GNA. The binding of PPT was inhibited by preincubation of the membrane with the lectins, suggesting that PPT binds to the glycoprotein epitopes of these lectins. PPT showed a binding pattern in the protein bands of BBM that was different from that of PPA. The enzyme activity of PPT measured with N-α-benzoyl-L-arginine ethylester was enhanced in the presence of the BBM fraction as well as with 0.2 M methyl α-D-mannoside, but not 0.2 M lactose. Conclusion The results indicate that mammalian pancreatic trypsins and chymotrypsin have carbohydrate-binding activity toward both glycoproteins possessing *N*-linked glycans and their component sugars, while trypsinogen binds glycoproteins and sugars differentially. Binding studies with the BBM fraction indicated the presence of glycoreceptors for trypsin and that the interaction with glycoreceptors enhances the trypsin activity. [1] Matsushita, H., Takenaka, M. and Ogawa, H. (2002) J. Biol. Chem. 277, 4680-86

(145) **Utility of Pseudoproteoglycan (pseudoPG) Probes that Simulate PG Supramolecular Structure**

Keiko Sato, Makiko Yamagami and Haruko Ogawa
Graduate School of Humanities and Sciences, Ochanomizu University, Tokyo 112-8610 Japan.

[Introduction] A proteoglycan (PG) monomer is a supermolecule consisting of glycosaminoglycan (GAG) chains attached to a core protein. PGs have signaling roles and cell modulatory functions in the extracellular matrix and at the cell surface. In this study, to elucidate the function of the PG higher-order structure, pseudoPGs that simulate the structure of a PG with GAG side chains attached to a linear polypeptide strand were prepared to develop probes and affinity adsorbents to search for and locate PG-binding substances. They were used to screen ligand proteins in extracts from rat brain. [Methods and Results] Probes. GAGs were coupled with poly-L-lysine (PLL) (Mw 15,000-30,000) by reductive amination (GAG-PLL) and biotinylated using NHS-biotin; then, the remaining amino groups were blocked by N-acetylation to obtain biotinyl poly-L-lysine (BPL)-GAG probes. The coupling of heparin (Hep) to a PLL (Hep-PLL) was detected by SDS-PAGE and gel-filtration HPLC. As a control, a BPL probe was prepared without GAGs. A biotin hydrazino-Hep (Bio-Hep) probe, which consists of a simple Hep chain coupled with biotin hydrazide and a neoproteoglycan (neoPG) probe, and Hep coupled to horseradish peroxidase (HRP-Hep) with the aid of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) were prepared for comparison. Adsorbents. Hep-PLL was immobilized to formyl-Sepharose by reductive amination to obtain Hep-PLL-Sepharose (bound Hep 1 mg/g gel). As a control, PLL-Sepharose without GAGs was prepared. For comparison, Hep-Sepharose was prepared by directly immobilizing Hep chains to amino-Sepharose using EEDQ (bound Hep 7 mg/g gel). Detection of Hep-binding proteins. HRP-Hep and BPL-Hep probes bound to more protein bands of rat brain extracts than biotin hydrazino-Hep probe did on Western blotting. Remarkably, several proteins in rat brain extracts specifically bound to either the BPL-Hep or the bio-Hep probes, indicating that the Hep-binding proteins exhibit specific recognition for a higher-order structure of PG. Separation by affinity chromatography. Extracts from normal rat brains were applied to affinity chromatography on columns of Hep-PLL-Sepharose or Hep-Sepharose. The proteins that specifically bound to either of the two adsorbents were identified by direct sequencing. One of the proteins that specifically bound to Hep-PLL-Sepharose was a multifunctional enzyme, cyclophilin A, while one that bound to Hep-Sepharose was a neurite promoting factor, amphoterin (HMG1). [Discussion] Host cyclophilin A has been reported to be involved in an initial process of HIV-1 attachment to macrophages and subsequent infection[1]. Our result indicates that a PG structure containing heparan sulfate is recognized by cyclophilin A at this stage, and pseudoPG

may serve as a more powerful inhibitor than conventional GAG probes for syndecan-HIV-1 interaction. This study indicates the utility of pseudoPG for the detection and separation of proteins and recognition of events that involve higher-order PG structures. [1)] Andrew C. S. Saphire et al. (2001) J. Virol. 75, 9187-9200

(146) **Cross-linking Studies of Concanavalin A and Dioclea Grandiflora Lectin with Synthetic Multivalent Carbohydrates**

Tarun K. Dam[1], Stefan Oscarson[2], Rene Roy[3], Frank Macaluso[1] and C. Fred Brewer[1]

[1] Albert Einstein College of Medicine, Bronx, New York, [2] Stockholm University, Stockholm, [3] Universite du Quebec a Montreal, Canada.

The Jack bean lectin concanavalin A (ConA) and the Dioclea grandiflora lectin (DGL) are Man/Glc-specific members of the Diocleinae subtribe. Both lectins bind with high affinity to the 'core' trimannoside moiety that is present in asparagine-linked carbohydrates, and both possess highly conserved binding sites and quaternary structures. Due to their multisubunit structures, both lectins can bind, cross-link and precipitate with multivalent carbohydrates. The present study is an investigation of the comparative binding and cross-linking properties of ConA and DGL with a series of synthetic divalent carbohydrates. The divalent analogs possess varying spacer groups between nonreducing terminal mannopyranoside residues. Hemagglutination inhibition, isothermal titration microcalorimetry and electron microscopy studies are used to characterize the binding and cross-linking interactions of the two lectins with the carbohydrates. The results demonstrate that despite their high degree of conserved binding specificity and quaternary structures, the two lectins exhibit differences in their cross-link activities with the divalent analogs. Electron microscopy results also show that increasing the distance between binding epitopes in the carbohydrates leads to a loss of observable, organized cross-linked structures with the two lectins.

(147) **Use of Synthetic Tetrasialosides for Modeling of Multivalent Interactions between Influenza Virus and Its Cellular Receptors**

Alexander Chinarev[1], Alexander Tuzikov[1], Alexander Sobol[1], Anne Imberty[2], Alexandra Gambaryan[3] and Nicolai Bovin[1]

[1] Shemyakin&Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia, [2] CERMAV-CNRS, Grenoble, France, [3] Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow, Russia.

To invade a host cell, influenza virus must find sialic receptors on cell membrane. Hemagglutinin (HA), trimeric glycoprotein covering virus surface, is responsible for the receptor recognition, multiple bonds between HA trimers and the receptors are needed for tight virus to cell attachment. Formally both intra- and inter-HA trimer modes are possible for this multivalent interaction, which of them actually occurs is not apparent. In order to clarify this we synthesized a set of tetrasialosides, [Neu5Acα-spacer-AC_n-$NHCH_2]_4C$, where AC_n - 'antennae' built of 1 to 6 aminocaproic residues and tested them as inhibitors of influenza virus receptor-binding activity. The substances with n=1-4 demonstrated low inhibitory potency, whereas the tetrasialosides with n=5-6 were in about two-three orders of magnitude more active. We built molecular mechanic models of the tetrasialoside molecules, among the models with coiled polyamide antennae appears to be more favorable by energy, and estimated size of the tetrasialosides in the calculated conformations. Alternatively, we measured diffusion coefficient of [Neu5Acα-spacer-AC_n-$NHCH_2]_4C$ molecules in aqueous solution using NMR-approach and calculate Stokes radii of the molecules arbitrary considering them as spheres. For all the tetrasialosides the radius value does not exceed ~30 A, which is in a good agreement with the size of coiled models. This justifies that in aqueous solutions the tetrasialoside molecules are compactly folded. Analysis of the obtained results and data on influenza virus structure brought us to the conclusion that the folded tetrasialoside molecules incapable of intra-trimer binding with virus both due to complex relief of HA surface and its extensive glycosylation. Moreover, in case of the intra-trimer binding Neu5Ac docking takes place at unfavourable angle. The inter-trimer binding is unaffected by these factors. Receptor-binding sites of neighbour HA trimers are separated 50 - 90 A, thus the coiled tetrasialoside molecules could easily be placed between HA trimers and adjust themselves for the multivalent interaction. The developed approach may be useful both for detailed understanding of the mechanism of virus to cell attachment and for designing of anti-adhesive therapeutics against influenza virus. This work is supported by ISTC grant #2464

(148) **Thermodynamic Analysis for the Sugar Recognition of Fbs1**

Shinya Hagihara, Kiichiro Totani, Ichiro Matsuo and Yukishige Ito

RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako-shi,

Saitama 351-0198, Japan, CREST, JST Kawaguchi 332-1102, Japan .

SCF complex is one of the most extensively studied ubiquitin ligases. It consists of Skp1, Cul1, Roc1 and an F-box protein that recognizes the target protein. Fbs1 is a recently discovered F-box protein, which was revealed to recognize high-mannose-type sugar chains[1]. Misfolded glycoproteins destined for degradation in endoplasmic reticulum are trimmed to $Man_8GlcNAc_2$ and transported from ER to cytosol to be degradated by ubiquitin-proteasome system[2]. Hence, Fbs1 is considered to recognize $Man_8GlcNAc_2$ or further trimmed form of N-glycan.

Pull-down analysis of the interaction between Fbs1 and N-glycoproteins revealed that Fbs1 recognizes the N-glycans containing a diacetylchitobiose structure with mannose residues. In addition, NMR studies and X-ray analysis of Fbs1 in complex with chitobiose have clarified the sugar recognition site of Fbs1[3]. However, quantitative analysis of the binding of Fbs1 to N-linked sugar chains has yet to be conducted. Herein, we report the thermodynamic analysis of the binding of Fbs1 to a series of high-mannose-type oligosaccharides using isothermal titration calorimetry (ITC). These experiments provided detail information on the mechanism for the sugar recognition of Fbs1.

We first evaluated the affinity of Fbs1 to $Man_9GlcNAc_2$, $Man_8GlcNAc_2$, $Man_8GlcNAc$, and $Man_3GlcNAc_2$. The thermodynamic parameters were obtained by fitting the ITC data to a binding model involving a single set of identical sites. The binding constant (Ka) for the complexation of $Man_8GlcNAc_2$ to Fbs1 was determined to be $3.2 \times 10^5\ M^{-1}$. In contrast, the binding of $Man_8GlcNAc$ was considerably weaker and not detectable by ITC. This result well-corroborates the crystal structure of chitobiose in complex with Fbs1. The affinity of $Man_9GlcNAc_2$ to Fbs1 (Ka = $3.2 \times 10^{5Å@}\ M^{-1}$) was similar to that of $Man_8GlcNAc_2$, whereas $Man_3GlcNAc_2$ showed stronger affinity to Fbs1 (Ka = $8.5 \times 10^{5Å@}\ M^{-1}$) than $Man_8GlcNAc_2$. These results suggest the steric repulsion between Fbs1 and the outer mannose residues of $Man_8GlcNAc_2$.

Subsequently, we measured the interactions between Fbs1 and the partial structures of $Man_3GlcNAc_2$. The binding of Man1-6Man$GlcNAc_2$ was marginally weaker than that of $Man_3GlcNAc_2$ (Ka = $3.3 \times 10^5\ M^{-1}$), while, Man1-3Man$GlcNAc_2$ drastically reduced affinity (Ka = $3.0 \times 10^{4Å@}\ M^{-1}$). These results indicate that the Man1-6 residue in the $Man_3GlcNAc_2$ plays a significant role for the binding with Fbs1. This fact was further examined by titrating the diantennary complex-type N-glycan (SiaGalGlcNAc1-2Man)$_2$Man$GlcNAc_2$ to Fbs1. The substituent at 2-position of Man1-6 in $Man_3GlcNAc_2$ reduced the affinity of (SiaGalGlcNAc1-2Man)$_2$Man$GlcNAc_2$ to Fbs1 (Ka = $2.4 \times 10^{4Å@}\ M^{-1}$), indicating that the 2-position of Man1-6 is strongly concerned with the sugar-recognition of Fbs1. Furthermore, the binding of chitobiose and branched mannnotriose to Fbs1 were not observed by ITC, suggesting that both of the GlcNAc residue at the reducing terminal and Man1-6 residue are necessary for the strong sugar-recognition of Fbs1.

Reference

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(149) **Design of the Blood Group AB Glycotope**

Elena Korchagina[1], Tatiana Pochechueva[1], Polina Obukhova[1], Anne Imberty[2], Robert Rieben[3] and Nicolai Bovin[1]

[1] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russian Federation,

[2] CERMAV-CNRS (affiliated with UniversitÈ Joseph Fourier), BP 53, F-38041 Grenoble Cedex 09, France, [3] Cardiology, Swiss Cardiovascular Center Bern, University Hospital, CH-3010 Bern, Switzerland.

Although the nature of the blood groups A and B has been comprehensively studied for a long time, it is still unclear as to what exactly is the epitope that is recognized by antibodies having AB specificity, i.e. monoclonal and

polyclonal antibodies which are capable of interacting equally well with the antigens GalNAcα1-3(Fucα1-2)Galβ (A trisaccharide) and Galα1-3(Fucα1-2)Galβ (B trisaccharide), but do not react with their common fragment Fucα1-2Galβ. We have supposed that besides Fucα1-2Galβ, A and B antigens have one more shared epitope. The trisaccharides A and B are practically identical from the conformational point of view, the only difference being situated at position 2 of the Galα residue, i.e. trisaccharide A has an NHAc group, whereas trisaccharide B has a hydroxyl group. We have hypothesized that the AB-epitope should be situated in the part of the molecule that is opposite to the NHAc group of GalNAc residue. In order to test this hypothesis we have synthesized a polymeric conjugate in such a way that the (A-)trisaccharide is attached to a polymer via the nitrogen in position C-2 of the GalNAcα residue. In this conjugate the supposed AB-epitope should be maximally accessible for antibodies from the solution, whereas the discrimination site of antigens A and B by the antibodies should be maximally hidden due to the close proximity of the polymer. Interaction with several anti-AB monoclonal antibodies revealed that a part of them really interacted with the synthetic AB-glycotope, thus confirming our hypothesis. Moreover, similar antibodies were revealed in the blood of healthy blood group 0 donors. Analysis of spatial models was performed in addition to identify the hydroxyl groups of Fuc, Galα, and Galβ residues, which are particularly involved in the composition of the AB-glycotope.

(150) **The Effects on the Expression of Human Immune Molecules on Human PMBCs by N-glycosylation Inhibitors**

Xiao-Lian Zhang[1], Juan-juan Wu[1] and Xiang Zhou[2]

[1] Department of Immunology, College of Medicine, Wuhan University, Wuhan 430071, P R China,

[2] College of Chemistry and Molecular Science, Wuhan University, Wuhan 430072, P R China.

Glycoprotein processing inhibitors, 1-deoxynojirimycin (DNM) and 1-deoxymannojirimycin (dMAN), are potential inhibitors of tumor metastasis and viral replication. However the effects of the inhibitors on human immune system are not well known. In this study, the effects on the expression of CD3, CD4, CD8, CD19, CD14 and HLA I molecules on human Peripheral Blood Mononuclear Cells (PMBCs) by DNM and dMAN were investigated. It was found that dMAN had remarkable inhibition effects on surface expression of CD3 and CD4 molecules, which are hallmarks on T cells. dMAN, much less toxic than Cyclosporin A (CyA), might have benefits to prevent binding of gp120 of HIV to CD4 molecules on human T cells. Corresponding author. Tel: +86-27-87331183; fax: +86-27-87336380; e-mail: zhangxl65@hotmail.com

(151) **Characterization of CD28 Glycosylation in T cells**

Mary A. Turner and Dorothy E. Lewis

Department of Immunology, One Baylor Plaza, Baylor College of Medicine, Houston, TX 77030.

CD28, a 44 Kd homodimer with five N-linked glycosylation sites is a key costimulatory molecule on T cells and is important for optimal T cell activation in immune responses. It interacts with ligands (B7, CD80 and CD86) on antigen presenting cells and until recently, it was thought that stimulation with CD28 enhanced T cell receptor (TCR) signaling but had no effect on its own. New data, however, show that certain CD28 monoclonal antibodies (mAb) can act in an agonistic manner, independent of stimulation via the TCR. One mechanism for this agonistic reactivity could be glycosylation-related conformational differences in cell surface CD28 influencing how the T cell responds to various stimuli. In earlier studies, we found an agonistic CD28 mAb (ANC28) which induces both activation (CD69 upregulation) and death (AnnexinV binding) of the T cell line, Jurkat. However, another T cell line, H9, showed only an increase in CD69 but no death. By Western blot analysis we observed differences in CD28 molecular weight (MW) from the two T cell lines. Under non-reducing conditions, Jurkat contained diffuse bands from 80-90Kd whereas H9 CD28 had fewer diffuse bands and these were of lower MW (50-60Kd). Another N-linked glycosylated protein, CD2, showed no differences between the two cell lines. To determine if the observed differences in CD28 MW were due to differential glycosylation, we used the glycosidases, N-glycanase F (PNGaseF) and endoglycosidase H (endoH). CD28 from Jurkat was digested by PNGaseF, but not by endoH, whereas CD28 from H9 cells was digested by both glycosidases. In the present studies, we used monensin and brefeldin A, two antibiotics that block the entry of N-linked glyco-proteins into the Golgi and neuraminidase, an enzyme that cleaves sialic acids. By Western blot analysis, treatment of cells with both monensin and brefeldin A caused a downward shift in CD28 MW from Jurkat, whereas CD28 from H9 was unaffected. Treatment of Jurkat T cells with neuraminidase also caused a downward shift in CD28 MW, whereas CD28 from H9 was unaffected. These results suggest that CD28 from Jurkat, but not H9, T cells contain more complex oligosaccharides, indicative of glycosyl maturation of the protein beyond the trans Golgi. We also tested whether activation of T cells with phytohemaglutinin (PHA) might induce differential glycosylation in CD28. Treatment of Jurkat T cells with PHA resulted in a modest downward shift in CD28 MW whereas CD28 from H9 was unaffected. These data suggest that differential glycosylation of CD28 is important for regulation of T cell activation and survival. Complex oligosaccharides are associated with the more mature CD28 protein, which can signal both activation and death. In contrast, less mature CD28, including that induced after activation, might favor only activation signal delivery.

(152) **Binding of Verotoxin 1 to Globotriaosyl Ceramide-Cholesterol Lipid Rafts Is Determined by Fatty Acid Content**

Radhia Mahfoud, Murugesapillai Mylvaganam, Beth Binnington Boyd and Clifford Lingwood

Research Institute, Division of Infection, Immunity, Injury and Repair, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada.

Verotoxins (VT) are a family of *Escherichia coli*-derived toxins, which have been associated with hemolytic uremic syndrome, the leading cause of acute pediatric renal failure, and hemorrhagic colitis. Verotoxin 1 (VT1) shows terminal galα1-4gal-dependent binding to the neutral glycosphingolipid, globotriaosylceramide (Gb_3). Our previous studies have shown that the VT1- Gb_3 binding is affected by the ceramide fatty acid chain length, hydroxylation and unsaturation. In this study, we have analyzed the binding of VT1 to detergent resistant microdomains - "lipid rafts"- prepared from a mixture of purified renal Gb_3/cholesterol. Artificial Gb_3/cholesterol rafts were generated and separated through a sucrose gradient containing ^{125}I-VT1. Rafts of Gb_3 containing short chain fatty acid (C16) showed 75% binding compared to native human renal Gb_3. No significant VT1 binding to either C18 or C20 fatty acid Gb_3 (6.6% and 3%, respectively) was observed. Long chain fatty acid Gb_3 (C22 and C24) showed 75% and 62.5% binding, respectively. Fatty acid a-hydroxylation did not significantly affect binding. A mixture of different species of Gb_3 (C16 (27%)+C18 (11%)+C20 (11%) +C22 (18 %)+C24 (33%)) was prepared according to the mass spectrometry analysis of the native Gb_3. Surprisingly, there was no VT1 raft binding for this mixture. Addition of α-hydroxylated C18 increased VT1 binding. Addition of a-hydroxylated C22 showed 100% binding compared to the native Gb_3. VT1 binding to the Gb_3 fatty acid isoform mixture showed a very significant increase upon removal of the C18 isoform (2 times compared to the native Gb_3). Removal of C20 had a less, but significant effect (100% binding compared to the native Gb_3). Our results indicate that, within the natural range, fatty acid chain length has a very important effect on verotoxin/ Gb_3 raft binding. The presence of a single isoform can determine maximum or minimum binding. Receptor function as assayed by lipid raft assay does not necessarily correlate with direct binding on TLC or microtitre plates. Our evidence indicates that within cholesterol rafts, a mere 2-carbon difference within the Gb_3 acyl chain can act as a 'switch' to define verotoxin binding.

(153) **Endoglycan is Expressed on Leukocyte Subsets with Modifications that Suggest Dual Functions as a Selectin Ligand and Chemokine Presentation Molecule**

Sheena C. Kerr, Claudia B. Fieger and Steven D. Rosen

Department of Anatomy, Program in Immunology, University of California, San Francisco, California, 94143-0452.

The process of leukocyte homing whereby circulating leukocytes leave the bloodstream and enter lymph nodes through specialized vessels called high endothelial venules (HEV) is essential for efficient immune surveillance. However, HEV-like vessels are also found at sites of chronic inflammation where they act as the primary means of tissue entry. Recently, work within our laboratory characterized a novel glycoprotein called endoglycan (gene name PODXL2), which is expressed on HEV. A chondroitin sulfate-modified sialomucin, endoglycan is a member of the CD34 family which includes CD34 and podocalyxin. Further characterization of this molecule demonstrated it can function as an L-selectin ligand and support lymphocyte rolling (Fieger et al. JBC 278: 27390, 2003). Here we report the expression of endoglycan on several leukocyte subsets as determined by flow cytometry. It is expressed on peripheral blood monocytes and represented on several human monocyte lines. Furthermore, expression was also detected on peripheral blood B cells, tonsillar B cells and several B cell

lines. The exact B cell subsets which express endoglycan are currently under investigation. In monocytes, the molecular weight of endoglycan indicates extensive modifications, which include the presence of chondroitin sulfate chains and sLex-related carbohydrates (HECA-452 reactivity). The latter feature is consistent with the possibility of selectin ligand function. Endoglycan in B cells also carries chondroitin sulfate modifications, although these cells appear to express the HECA-452 epitope only after cellular activation. Potentially, this could allow endoglycan to mediate secondary leukocyte-leukocyte interactions between rolling and adhered leukocytes or mediate B-cell interactions with activated endothelium via the vascular selectins. Chondroitin sulfate (CS) chains have been shown to interact with chemokines, an activity that is influenced by the level of sulfation present on the molecule (Kawashima et al. JBC 277: 12921, 2002). Over-sulfated CS chains such as CS-E (GlcA-GalNAc 4S, 6S), generated from CS-A (Glc-GalNAc-4S) by the activity of the B cell recombination activating gene-associated gene (BRAG), have been shown to have a higher affinity for chemokine binding. All the endoglycan positive B cell lines tested thus far by RT-PCR have been shown to express BRAG, raising the possibility that endoglycan expressed in B cells could be modified with CS-E. Chemokine protection assays using recombinant endoglycan have demonstrated that the molecule is capable of interacting with several homing and inflammatory chemokines. This work is being extended with a view to B-cell homing chemokines and to eluicidating the exact modifications required for presentation. In summary, this is the first report that endoglycan is expressed on human peripheral blood monocytes, B cells and tonsillar B cells, thus identifying a new leukocyte receptor that could play multiple functions in lymphocyte homing under both homeostatic and inflammatory conditions.

(154) **Isoforms of Glucosidase II Differently Recognize Both Oligosaccharides and Protein Moieties of High-mannose Bearing Glycoproteins**

Masahiko Ikekita and Masaaki Toyoda
Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba, Japan.

Processing of the Asparagine (N)-linked oligosaccharides is processed by the removal of the terminal glucose residue by the glucosidase I. and two inner glucose residues are then removed by the Glucosidase II (GII). Some of the mannose residues from Man9GlcNAc2 are trimmed by ER (endoplasmic reticulum)-mannosidases, and then the trimmed glycoproteins are transported to the Golgi apparatus. It has been proposed that N-linked oligosaccharides are important the quality control mechanism of the glycoproteins. When the parts of the polypeptides of the glycoproteins that are linked to the Man9GlcNAc2 are unfolded in the ER, the oligosaccharides of the glycoproteins interact with the UDP-Clc: glycoprotein glucosyltransferase (UGGT). UGGT serves as the folding sensor by selectively reglucosylating those polypeptides that have not acquired their native folded conformation. Calnexin, chaperone molecule in ER, recognizes the Glc1Man9GlcNAc2 on the proteins and folds part of the polypeptide of the glycoprotein. Recently, regarding the interaction of GII with the glycoproteins, it was reported that the higher molecular weight isoform of GII (GII-H) was associated with CD45 by a lectin-like interaction. We investigated the detailed interaction of GII with glycoproteins containing oligomannosidic oligosaccharides by the use of the purified GII and prepared columns including ribonuclease B (RNase B), thyroglobulin (TG), and ovalbumin (OVA). Here, we show that the mannose-specific binding activities of GII to glycoproteins containing oligomannosidic saccharides requires both oligosaccharides and the protein moieties of the glycoproteins, and that two isoforms of GII, GII-H and -L (lower molecular weight isoform of GII), have different recognition patterns of glycoproteins. We examined the mannose-specific binding of GII. The GII-L and GII-H bind to various treated RNase B-columns by mannose-specific binding activities, but they did not bind to its glycopeptides and oligosaccharides columns. Furthermore, GII-H binds to variously treated TG columns by mannose-specific binding, but it did not bind to its glycopeptides and oligosaccharides columns. GII-L and -H strongly bind to variously treated RNase B- and Tg- columns, respectively. Additionally, GII-H and -L did not bind to the column prepared from RNase B that is digested with proteinase K, and GII-H did not bind to the column prepared from Tg that is digested with trypsin or proteinase K. Taken together, these results show that the mannose-specific binding of GII-H and -L to glycoproteins containing oligomannosidic saccharides requires both oligosaccharides and protein moieties of the glycoproteins, and that GII-H and -L have different recognition domains for glycoproteins. These properties of GII may be related to the recognition of ligands that GII hydrolyzes. We were not able to determine whether the difference of the mannose-specific binding of GII-H and -L depends on the differences among oligosaccharides or the protein moiety, or both the oligosaccharides and protein moieties.

(155) **Structural Basis of Venom citrate-dependent Heparan Sulfate-mediated Cell Surface Retention of Cobra Cardiotoxin A3**

Shao-Chen Lee[1], Hong-Hsiang Guan[1], Chia-Hui Wang[1], Wei-Ning Huang[1], Chun-Jung Chen[2] and Wen-guey Wu[1]
[1] Institute of Bioinformatics and Structural biology, National TsingHua University, Hsinchu 30043, Taiwan., [2] Biology Group, Research Division, National Synchroton Radiation Research Center, Hsinchu 30077, Taiwan..

Anionic citrate is a major component of venom, but the role of venom citrate in toxicity is poorly understood other than inhibitory effect on the cation-dependent action of venom toxins. By using immobilized Chinese hamster ovary (CHO) cells in microcapillary tubes and immobilized heparin in sensor chips, we demonstrate that heparan sulfate (HS) mediated cell retention of major cardiotoxin (CTX) from Taiwan cobra, i.e., CTX A3, require citrate-dependent oligomerization of CTX A3 on membrane surface. X-ray determination of CTX A3-heparin hexasaccharide complex structure reveals a molecular mechanism of citrate-induced dimerization of CTXs by interacting with Lys31 near the tip of loop II to stabilize the hydrophobic contact of the dimeric CTX A3 at the functionally important loop I and loop II regions. Our result suggests a novel role for venom citrate activity and identify specific sulfation pattern of HS in binding to CTX A3 for exerting cytotoxicity. It also reveals a structural model on how HS-CTX interaction could stabilize the CTX-lipid membrane interaction.

(156) **Mechanism of Inhibition of the Metalloprotease Meprin α and β by Mannan-binding Protein**

Makoto Hirano[1], Bruce Y. Ma[1], Makoto Baba[1], Ryuuya Murai[1], Nana Kawasaki[3], Shogo Oka[1], Nobuko Kawasaki[2] and Toshisuke Kawasaki[1]
[1] Department of Biological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Japan
[2] School of Health Sciences, Faculty of Medicine, Kyoto University, Japan
[3] Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Japan.

Å@ Mannan-binding protein (MBP) is a C-type animal lectin which is known as a host defence factor involved in innate immunity and recognizes mannose, fucose and N-acetylglucosamine residues. Although some MBP exogenous ligands have been reported, little is known about the endogenous ligands. In the present study, we report that both meprin α and β (meprins) as novel MBP endogenous ligands have been identified using affinity chromatography and mass spectrometry. Meprins are membrane-bound and secreted zinc metalloproteases extensively glycosylated and highly expressed in kidney and small intestinal epithelial cells, leukocytes and certain cancer cells. There are two types of meprin subunits, α and β, which form disulfide-bound homo- and hetero-oligomers, further oligomerization is mediatd by non-covalent interactions. Meprins are capable of cleaving growth factors, extracellular matrix proteins, and biologically active peptides. Deglycosylation experiments with N-glycosidase F and endoglycosidase H indicated that the MBP ligands on meprins are high mannose- or complex-type oligosaccharides. The interaction of MBP with meprins resulted in significant loss of the matrix-cleaving activity of meprins. Our results suggest that N-linked oligosaccharides on the meprins are involved in the optimal enzymatic activity and MBP is an important regulator for the modulation of the localized meprin proteolytic activity via N-glycan-binding. Because meprins are known to cleave basement membrane components, MBP, which functions as a natural and effective inhibitor against meprins, may contribute to tumor progression by facilitating migration, intravasation, and metastasis of carcinoma cells.

(157) **Carrier and Barrier Functions of Proteoglycans in Cellular Uptake of Charged Macromolecules**

Staffan Sandgren, Anders Wittrup and Mattias Belting
Department of Cell and Molecular Biology, Section of Cell and Matrix Biology, Lund Univeristy, BMC, C13, S-221 84 Lund, Sweden.

Recent studies demonstrate uptake of macromolecular polyanions (nucleic acids and glycosaminoglycans), as well as polycations (cationic lipids and polybasic peptides) into mammalian cells. In many cases these evolutionary

conserved transport pathways are exploited by microbial intruders, such as viruses and bacteria. In the new era of gene therapy and gene silencing, which are based on intracellular delivery of DNA and RNA, it has become increasingly important to elucidate the molecular mechanisms of these transport pathways. Here, we demonstrate a regulatory function of cell-surface proteoglycans (PGs) in the uptake of the macromolecular polyanions DNA and heparan sulfate (HS) by mammalian cells. Employing flow cytometry, confocal microscopy, and reporter gene assay, we show that free DNA and HS share a common transport pathway that exhibits saturable kinetics and temperature-dependency. Interestingly, PG-deficient cells show increased polyanion uptake as compared with wild-type cells, suggesting that PG-derived, endogenous polyanions significantly repell exogenous polyanion uptake. When DNA and HS form complexes with polybasic peptides, such as HIV-Tat and the antimicrobial peptide LL-37, polyanion uptake increases several-fold, a process that is dependent on a net positive charge of the complexes. Internalization of such complexes is strictly dependent on the expression of endogenous PG, and involves cholesterol-rich, lipid raft membrane domains. These data are consistent with previous work, demonstrating an involvement of the cell-surface HSPG glypican-1 in polyamine uptake. Finally, we demonstrate that cells secrete endogenous components with the intrinsic ability to efficiently stimulate polyanion uptake. Interestingly, polyanion uptake mediated by secreted componets also depends on cell-surface PGs. The isolation and identification of active components is the focus of our ongoing studies, and preliminary data suggest that proteins with high heparin affinity are responsible for the effects on polyanion uptake. To conclude, our data show that the ubiquitous distribution of PGs at the surface of a typical cell membrane regulates the entry of macromolecules. Membrane passage efficiency is thus largely the result of macromolcular net charge as well as the status of the PG biosynthesis machinery. The physiological role of this transport pathway is ill-defined, but it may be speculated that endogenous polycation-polyanion complexes (i.e. transcription factors and nucleic acids) traffic between cells in a process that is regulated by cell-surface PG.

(158) **Carbohydrate-binding Characteristics of Human C-reactive Protein**

Reiko T. Lee and Yuan C. Lee
Dept. of Biology, Johns Hopkins Univ. 3400 N. Charles St., Baltimore, MD 21218.

Human C-reactive protein (CRP) is a proto-type acute phase reactant, which is often used to gauge the presence of inflammation. CRP can eliminate some microorganisms in a fashion similar to IgG, by initiating classical complement cascade after binding to multivalent ligands. Structurally, CRP belongs to pentraxin family, having five monomers associating non-covalently in a ring form. One face of pentameric ring contains all the calcium-binding sites, two sites per monomer in close proximity to each other. Phosphoryl choline (PC), the best ligand for CRP, is bound near the calcium site, with phosphate group directly coordinating with both calcium atoms. Conformation in the calcium-binding area undergoes change depending on the presence or absence of calcium. In addition, there is a slightly denatured, but physiologically important, form of CRP, known as neoCRP or mCRP, which is formed, for instance, when CRP is adsorbed onto a plastic surface. CRP binds at least three types of ligands: 1) PC and related structures; 2) poly-cationic compounds, such as poly-lysine and protamine sulfate; 3) carbohydrates containing galactose and related structures. We developed ligand-binding assays based on europium-labeled reporter ligands that are applicable to all three ligand groups. The native CRP assay uses polyethyleneglycol-assisted precipitation of CRPóligand complex, and neoCRP assay is carried out with CRP that is immobilized onto 96-well microplate. The following major conclusions were obtained from direct and inhibition assays using europium-labeled PC-BSA, poly-L-lysine, lactose-BSA, Gal6P-BSA, and Galb(1,3)GalNAc-BSA. 1) Binding of PC requires calcium, while neutral sugars are bound in the absence of calcium. Poly-cations are bound with or without calcium. 2) Gal6P is the best carbohydrate ligand, and it can be bound with or without calcium. PC and Gal6P share the same phosphate-binding site, with choline and galactose moieties occupying either side of phosphate. Binding of Gal6P in the presence of calcium is approximately 10-fold stronger than in the absence of calcium. 3) Neutral sugars are much weaker ligands than Gal6P. However, we showed that BSA conjugate containing Galb(1,3)GalNAc (one of the best disaccharide inhibitors) has sufficient affinity to be bound to both native CRP and neoCRP. Gal6P is the best inhibitor for the binding of Galb(1,3)GalNAc-BSA. 4) Unlike PC which is bound in a wide pH range, binding of carbohydrate ligand, whether neutral or phosphorylated, has pH optimum around 6.5 above which binding decreases steadily, suggesting that the binding site for galactose and disaccharides becomes masked above neutral pH. 5) Protein structure of neoCRP appears to be more fragile than native CRP in the acidic region. Binding of all ligands, including PC-BSA, declined in a parallel fashion below pH 6, having no binding activity left at pH 4.5. In contrast, native CRP maintains active conformation down to pH 4.5.

(159) **Multivalency and the Mode of Action of Bacterial Sialidases**

Geert-Jan Boons, Smita Thobhani and Brian Ember
Complex Carbohydrate Research Center, University of Georgia, 315 Riverbend Road, Athens, Georgia 30602.

Multivalent binding events, in which multiple ligands on one entity simultaneously interact with multiple receptors on a complementary entity, are widespread in nature. This type of interaction has been demonstrated to be mechanically and functionally distinct from its monovalent alternative and relatively commonplace in carbohydrate-mediated biological events. The best-studied manifestations of multivalency include dramatic increased functional affinities, enhanced or altered selectivities, and initiation of cell signaling events. We have discovered a new manifestation of multivalency and demonstrate for the first time that bacterial sialidases, which contain a catalytic domain together with one or more carbohydrate-binding domains, are able to hydrolyze polyvalent substrates with much greater catalytic efficiency than monovalent counterparts. The striking difference in enzymatic activity displayed by these enzymes is explained by invoking a model wherein the catalytic- and lectin domains interact simultaneously with the polyvalent substrate. These findings have been exploited in the design of a novel polyvalent inhibitor of the sialidase of Vibrio cholerae that targets the lectin domain. This inhibitor is the first of its type in that it is not based on a sialic acid related scaffold and demonstrates a simple way of engineering exquisite selectivity for inhibitors of modular enzymes that possess a catalytic domain together with one or more binding domains.

(160) **Structural Basis of Sugar Recognition by the Cargo Receptor VIP36**

Yukiko Kamiya[1], Yoshiki Yamaguchi[1], Ichiro Matsuo[2], Yukishige Ito[2], Masayasu Toyomoto[3], Naoki Matsumoto[3], Kazuo Yamamoto[3] and Koichi Kato[1]
[1] Graduate School of Pharmceutical Sciences, Nagoya City University, Japan, [2] RIKEN, Japan, [3] Graduate School of Frontier Science, The University of Tokyo, Japan.

VIP36 and ERGIC-53 are intracellular lectins that act as cargo receptors and play crucial roles in vesicular transport of glycoproteins. Although VIP36 and ERGIC-53 have been reported to recognize glycoproteins carrying high-mannose type oligosaccharides, structural basis of the carbohydrate recognition by these cargo receptors has been poorly understood. Here we report an NMR study of interactions between VIP36 and oligosaccharides. For this purpose, we used synthetic oligosaccharides and a recombinant carbohydrate recognition domain of VIP36 (designated as VIPCRD) expressed by *E. Coli*.

Inspection of ^{1}H-NMR spectral data of a high-mannose type oligosaccharide, $Man_9GlcNAc_2$, titrated with VIPCRD showed that the trimannose residues in the D1 arm, Manα1-2Manα1-2Man, form the epitope recognized by VIP36. Conformation of the ligand bound to VIPCRD was determined based on the transferred NOE data, which indicated that the trimannose residues are accommodated in VIPCRD exhibiting the typical Manα1-2Man linkage conformation. The sugar-binding site of VIPCRD was identified by use of ^{13}C- and ^{15}N-labeled VIPCRD. Chemical shift perturbation data showed that the epitope-binding site locates on a concave face of the β-sandwich. VIPCRD requires Ca^{2+} for glycoprotein-binding, which is enhanced by lowering pH. NMR spectral analyses revealed that conformation and dynamics of VIPCRD are altered in a Ca^{2+}- and pH- dependent manner. On inspection of these data, biological significance of the sugar-binding mode of VIP36 will be discussed in comparison with leguminous and other animal lectins.

This work was supported by CREST/JST.

(161) **The Biological Evaluation of a Novel Glycosaminoglycan**

Rhona M. Duane[1], Ciaran Lynch[1], Nigel Pitt[2], Helena Bradley[2], Alan O' Brien[2], Paul V. Murphy[2] and Kathy M. O' Boyle[1]
[1] Department of Pharmacology, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland , [2] Centre for Synthesis and Chemical Biology, Department of Chemistry, Conway Institute of Biomolecular an

Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland.

Glycosaminoglycans (GAGs) are a family of carbohydrates composed of highly charged polysaccharide chains which interact with physiologically important glycoproteins including, fibronectin and fibroblast growth factor (FGF). The biological effects resulting from these interactions include tissue repair, angiogenesis, embryogenesis, blood clotting and cell migration/adhesion. Compounds that alter endothelial cell growth are of particular interest in the development of angiogenesis modulators. The purpose of this study was to identify from a library of novel glycomimetics a compound which interferes with glycoprotein-GAG interactions and which can effect the growth of bovine aortic endothelial cells (BAECs). A structurally diverse series of 40 saccharide derivatives were synthesized by Dr. Paul Murphy and colleagues and evaluated for their effects on the growth of BAECs. Heparin-albumin was found to inhibit the survival of the cells by 37% at a concentration of 10µg/ml after 24 hours, as determined using a methyl tetrazolium (MTT) assay. Under identical conditions, a number of the novel saccharide conjugates were found to mimic the effect of heparin-albumin. Thioglucamide was found to be one of the most potent inhibitors of cell survival showing 18% inhibition at 35µM. The effects of thioglucamide and heparin-albumin on absolute cell number were also studied using cell-counting experiments. Thioglucamide (36% after 24h) proved to be more potent than indicated by the MTT assay and initially reduced the BAEC number to a greater extent than heparin (30% after 24h). However the effects of heparin-albumin were more prolonged (25% after 72h) than the saccharide derivative whose inhibitory effect was lost after the same time period. In another assay to determine the effect of the saccharide derivatives on the proliferation of BAECs, thioglucamide was seen to inhibit the uptake of [^{3}H] thymidine (19% after 24h). Heparin-albumin also inhibited thymidine uptake (25% after 24h). None of the glycoconjugates reduced the proliferation of mouse mammary epithelial cells, nor did any alter gross cell morphology. These results support the proposal that the reduction in BAEC survival by saccharide conjugates such as thioglucamide is a result of the inhibition of cell proliferation rather than through the induction of cytotoxicity. The ability of the novel glycomimetic agents to compete with heparin for binding to FGF was assessed using an ELISA to determine the possible mechanism by which the glycoconjugates could alter growth of the endothelial cells. Of the novel saccharides tested, thioglucamide was found to have the greatest inhibitory effect on heparin-FGF binding causing a maximum inhibition of 62% at 346µM. Heparin and several other of the saccharide derivatives also inhibited FGF-heparin albumin binding to varying extents. However, there was no correlation between inhibition of BAEC survival and inhibition of FGF binding to heparin-albumin. This study has lead to the identification of a novel saccharide conjugate that can both interfere with FGF-heparin binding and that can inhibit endothelial cell growth. It is, therefore, of interest as a potential angiogenesis inhibitor. This work was supported by IRCSET, Bioresearch Ireland and the Programme for Research in Third-Level Institutions (PRTCI) administered by the HEA.

(162) **Theoretical Studies of Binding of Mannose-binding Protein to Monosaccharides**

Sachiko Aida-Hyugaji[1,2], Keiko Takano[2], Toshikazu Takada[3], Haruo Hosoya[2], Naoya Kojima[4], Tsuguo Mizuochi[4] and Yasushi Inoue[1]

[1] Information Technology Center, Tokai University, 1117 Kitakaname, Hiratsuka 259-1292, Japan, [2] Graduate School of Humanities and Sciences, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan, [3] Foundational Research Laboratories, NEC Corporation, 34 Miyukigaoka, Tsukuba 305-8501, Japan, [4] Faculty of Engineering, Tokai University, 1117 Kitakaname, Hiratsuka 259-1292, Japan.

Mannose-binding protein (MBP) is a member of the family of C-type (Ca^{2+}-dependent) lectins. MBP plays a crucial role in human innate immunity system through the recognition of mannose. It activates the lectin pathway of the complement system and acts as an opsonin. Moreover, it is reported that MBP inhibits infection to human cells with HIV in vitro. In this study, binding properties of MBP to sugar residues are discussed based on ab initio molecular orbital (MO) calculations. The electronic structures of MBP, sugar residues, and their complexes are described with reference to experimental results, e.g., X-ray crystallography and binding assays, in order to clarify the recognition mechanism of MBP to sugar residues and to predict the binding properties of sugar residues.

We calculated the binding energies between MBP and six sugar residues, which are mannose, glucose, N-acetylglucosamine, fucose, galactose, and N-acetylgalactosamine, using cluster models constructed. On the assumption that the coordination of Ca^{2+} significantly affects the electronic structure of MBP, the calculations were carried out in order to investigate the interactions between a cluster model of MBP with Ca^{2+} and each of the six sugar residues. The calculated binding energies indicate that MBP has an affinity for N-acetylglucosamine, mannose, fucose, and glucose rather than galactose and N-acetylgalactosamine, which is well consistent with the biochemical experimental results.

Next, we calculated electrostatic potential (ESP) iso-surfaces, molecular orbitals, net charges and atom distances. ESPs at the binding site of four monosaccharides having binding properties are complementary to that of MBP, but those of other two are not. It suggests that ESP is one of the essential determinants of sugar selectivity of MBP. A vacant frontier orbital localized at binding site of MBP and an in-phase occupied frontier orbital of mannose were found. Upon complexation, the frontier orbital of mannose is affected by Ca^{2+} and mannose can interacts with MBP through orbital interaction. Net charges and atom distances in MBP-mannose complex indicate the existence of four hydrogen bonds between amino acid residues of MBP and oxygens of mannose.

We conclude that MBP recognizes sugar residues in the two stages. At long range electrostatic potential dominantly plays an important role in sugar selectivity of MBP. At short range, in addition to the electrostatic potential, orbital interactions and hydrogen bonds, which is one of the electrostatic interactions, are important in stabilization of complexes of MBP and the selected sugar residues.

(163) **Lectin/carbohydrate Recognition Mediate *Trypanosoma cruzi* Infection of Endothelial Cells**

Fernanda D. Fajardo[1], Wagner B. Dias[1], MÙnica F. Souza[1], Elaine R.A. Barbosa[1], Mourielle F. Girard[2], Bernard Bouteille[2], JosÈ O. Previato[1], Lucia MendonÁa-Previato[1] and Adriane R. Todeschini[1]

[1] Instituto de Biof'sica Carlos Chagas Filho, CCS, Bloco G, Universidade Federal do Rio de Janeiro, Ilha do Fund,,o,1944.970, Rio de Janeiro, RJ, [2] Institut d'EpidÈmiologie Neurologique et de Neurorogie Tropicale, Limoges, France..

Communication between pathogen and host cells directs modulation of the host cell environment and immune escaping strategies influencing the outcome of infection. Initiation of communication between *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, and mammalian cells requires contact of parasite molecules (soluble or membrane boud) with host ligands. Ours studies demonstrated that cross talk between *Trypanosoma cruzi* and host cells may be mediated by molecules of the *trans*-sialidase (TS) family. *Trypanosoma cruzi*, expresses on its surface a sialic acid-binding lectin, containing a β-galactoside recognition site know as inactive *trans*-sialidase (iTS). iTS behaves as lectins that binds and triggers contact-dependent signals to cells of the host innate and adaptive immune system during *Trypanosoma cruzi* infection. iTS binds α2,3-sialic acid on CD43 from host CD4+ T cells and initiates co-stimulatory responses that increase mitogenesis, cytokine secretion, and promote rescue from apoptosis. As the vascular endothelium is an early target to parasite invasion and is strongly involved in the inflammatory process, in this work we studied the effect of TS on human bone marrow endothelial cells (HBMEC). We demonstrate that, iTS can physically interacts with α2,3-binding sialic acid containing molecules on human bone marrow endothelial cells (HBMEC) triggering NF-κB activation, expression of adhesion molecules and, blocking apoptosis induced by grown factor deprivation. HBMEC activation increases *Trypanosoma cruzi* trypomastigotes attachment to and invasion of endothelial cells. Further, iTS may play a major role in the inflammatory response during *Trypanosoma cruzi* infection, since it modulates leukocyte adhesion to endothelial cells. Taken together our findings suggest that molecules of *trans*-sialidase family play a role in the host/parasite interaction behaving either as receptors or ligands for parasite/host interactions, resulting in the attachment to and invasion of host cells, and immunoregulation, enabling *Trypanosoma cruzi* replication and establishment of infection. Supported by CNPq (PRONEX), FAPERJ, HHMI, CAPES/COFECUB, TWAS.

(164) **Galectins-1 and 3 Trigger T Cell Apoptosis Through Overlapping, Non-identical Complements of Glycoprotein Receptors**

Brianna N. Stillman[1], Daniel K. Hsu[2], C. Fred Brewer[3], Fu-Tong Liu[2] and Linda G. Baum[1]

[1] Dept. of Pathology and Laboratory Medicine, UCLA School of Medicine, [2] Dept. of Dermatology, UC Davis School of Medicine, [3]

Departments of Molecular Pharmacology, and Microbiology and Immunology, Albert Einstein College of Medicine.

Galectins-1 and 3 regulate T cell apoptosis. Intracellular expression of gal-3 inhibits apoptosis of T cells following apoptotic stimuli, while extracellular gal-1 or gal-3 directly induce apoptosis of T cells. We have previously shown that gal-1 binds to CD7, CD43, and CD45 to trigger T cell apoptosis. However, the complement of receptors that gal-3 binds to trigger T cell death is not known. I have identified CD29, CD43, CD45, and CD71 as major gal-3 receptors on T cells, and have investigated the roles of these glycoproteins in triggering gal-3 death. CD29 and CD43 were not required for gal-3 death, as gal-3 induced apoptosis in CD29 null Jurkat and CD43 null CEM T cell lines with the same efficiency as wild-type cells. Since it has been suggested that CD7 and CD29 associate and contribute to gal-3 death, I investigated whether CD7 is required for gal-3 death. In contrast to gal-1, CD7 is not required for gal-3 death, as the CD7 null Hut 78 T cell line is susceptible to gal-3. Unlike the CD7, CD29, and CD43 null T cell lines, a CD45 null Jurkat T cell line demonstrated reduced susceptibiltity to gal-3, suggesting that CD45 contributes to the gal-3 death signal. Studies investigating the role of CD71 are in progress. We have found that gal-1 and gal-3 bind to an over-lapping, non-identical complement of T cell surface glycoproteins in a carbohydrate dependent manner to trigger death. Thus, gal-1 and gal-3 are two galectin family members that induce T cell apoptosis through non-identical upstream events.

(165) **Identification of Multiple Tissue-Specific Spice Variants of Human HARE - The HA Receptor for Endocytosis**

Edward N. Harris and Paul H. Weigel

Department of Biochemistry & Molecular Biology, University of Oklahoma HSC, Oklahoma City, OK 73104.

The human Hyaluronic Acid Receptor for Endocytosis (hHARE; also called Stabilin-2) is the predominant endocytic clearance receptor for circulating hyaluronan (HA) and other glycosaminoglycans (GAGs) that originate from the ECM of tissues throughout the body. HARE is highly expressed in the endothelial cells of lymph node, spleen, and liver. The full-length human hare cDNA (7653 bp) encodes a glycoprotein of 2551 amino acids that migrates in SDS-PAGE to about 315 kDa on a 5% gel. Immunopurification of hHARE from spleen revealed that HARE exists as two receptor isoforms (315 kDa and 190 kDa) that are likely the result of differential proteolytic cleavage of the full-length protein. When expressed in 293 Flp-In cells, the 190 kDa hHARE binds to HA with high affinity. It also binds to chondroitin, chondroitin sulfates A, C, D, and E, but not to keratan sulfate, heparin sulfate or heparin. The human *hare* gene, located on the q arm of chromosome 12, spans ~180 kb and contains 69 exons of similar length. Since the full-length protein binds to multiple ligands and is encoded by many exons, we predicted that alternative splice variants exist in lymph node, liver, spleen, and other tissues where HA/GAGs are abundant or have important biological functions. Using cDNA pools prepared from spleen and lymph node mRNA and a battery of primer pairs, we used PCR to amplify and identify potential *hare* splice variants. So far, we have identified and sequenced eight splice variant candidates; six from spleen and two from lymph node. Of these eight splice variants, three cDNAs encoding the full-length HARE variant were then prepared, cloned into mammalian expression vectors, and expressed in 293 Flp-In cells. Two of the eight splice variants contained frameshifts caused by a splice junction within a codon sequence. If the frameshifted cDNAs are expressed, both putative proteins should be soluble and contain extracellular domain regions of HARE with unique C-termini. Preliminary experiments with the shortest of the splice variants, which contains only 7 of the 69 exons, suggest that the protein product does not bind to HA; we have not yet examined binding to other GAGs. We hypothesize that many more variants are yet to be discovered in lymph node, spleen, and other untested tissues, and that these HARE spice variants may play a variety of important roles in GAG function and homeostasis. (Supported by NIH grant GM69961 from the National Institutes of General Medical Sciences)

(166) **The GAG Binding of an Auto-antigen Explains the Joint Specificity and Distal Joint Involvement of Rheumatoid Arthritis.**

Daniel R. Studelska, Fei Shih, Kari Giljum, Paul M. Allen and Lijuan Zhang

Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110.

The K/BxN T cell receptor transgenic mouse provides a robust model of rheumatoid arthritis (RA). Inflammation is due to the autoimmune recognition of glucose 6-phosphate isomerase (GPI), as shown by the ability of affinity-purified anti-GPI antibodies from K/BxN mice to elicit the disease in lymphocyte-deficient recipient mice. GPI has been localized to the cartilage surface of normal ankles, but the enzyme is ubiquitous in cells and in the circulation, and the confinement of RA inflammation to the synovial joints is mysterious. As in the human disease, there are pronounced differences in the degree to which different joints are afflicted. In the K/BxN mouse, paws are affected more than knees, and hips are essentially spared. We have isolated the predominantly chondroitin sulfate GAGs from these joints in K/BxN and control mouse strains and quantified them to use equimolar amounts in gel-shift assays with ^{125}I-GPI. We discovered that GAGs isolated from paws are more potent at perturbing the electrophoretic mobility of GPI than GAGs isolated from knees. This result suggests that the joint-specific inflammation characteristic of RA depends on the GAG binding of this auto-antigen. In view of the importance of antigen access to the disease process, we surmised that glucuronic acid residues at the nonreducing end of the GAG chains might bind in the catalytic site of GPI. We found that fl-glucuronidase treatment substantially reduced the paws-over-knees GAG effect on GPI gel mobility. This suggests that GAGs beginning with glucuronic acid are necessary for GPI-GAG binding and are an abundant feature of the GAGs coating the articular cartilage of paws. Finally, we tested the ability of substrates or inhibitors of GPI to compete with the gel-shift effect of paws GAGs. We discovered a marked effect at about 10-100 times the Kd or Ki of these compounds for the enzyme. These results support the involvement of the enzyme active site in paws GAG binding and suggest that the GAG binding to GPI is relatively high affinity. The joint specificity and preference for distal joints of K/BxN RA may be a simple function of the amount of GPI tethered to the joint surface. The relatively high density and low turnover of auto-antigen in joints may be required to pass the threshold for inflammation.

(167) **Interaction of Ebola virus Glycoprotein with Human Macrophage Galactose-Type C-type Lectin**

Katsuaki Usami[1], Hideyuki Takeuchi[1], Kouki Fujioka[1], Ayato Takada[2,3], Yoshihiro Kawaoka[2,3,4] and Tatsuro Irimura[1]

[1] Laboratory of Cancer Biology and Molecular Immunology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, [2] Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, The University of Tokyo, [3] CREST, Japan Science and Technology Corporation, [4] Department of Pathobiological Sciences, University of Wisconsin.

[Background and Aim]

Ebola virus glycoprotein (EboGP) is highly glycosylated and contains both *N*- and *O*-glycans. We previously demonstrated that human macrophage galactose-type C-type lectin (hMGL) acts as an attachment factor for Ebola viral entry. However, structural features of EboGP responsible for this binding were unclear. In the present study, we modified EboGP glycans and tested their interaction with hMGL.

[Methods]

The virus-like particles with surface glycoproteins (VLP-EboGP) were purified from supernatants of 293T cells co-transfected with a plasmid encoding EboGP and matrix protein (VP40). These particles showed remarkably similar morphology to filovirus virions. Recombinant hMGL (rhMGL), corresponding to the extracellular domain was expressed in *E.coli* and purified by affinity chromatography on Galactose-Sepharose. VLP-EboGP, before and after modification of its carbohydrate moieties, was tested for its interaction with rhMGL by ELISA and Western blotting analyses.

[Results and Discussion]

When VLP-EboGP was treated with PNGase F, its binding to rhMGL did not change, whereas its interaction with PHA-L_4 was completely abolished after the same treatment. The binding of VLP-EboGP to rhMGL slightly increased after treatment with PNGase F and β-galactosidase or neuraminidase, and slightly decreased after treatment with PNGase F and ST3Gal II. Similar changes were observed in the binding to VVA-B_4. These results suggest that rhMGL binds EboGP through interaction with its *O*-glycans, especially Tn antigens (GalNAcα-Ser/Thr). The structures of EboGP *O*-glycans should be influenced by the host cell in which virions are formed. The ability of EboGP to interact with hMGL is likely to determine the virulence of Ebola virus.

(168) **Mouse Heat Shock Protein 70 Recognizes Negatively Charged Glycolipids and Phospholipids**

Yoichiro Harada[1], Chihiro Sato[1,2] and Ken Kitajima[1,2,3]

[1] Graduate School of Bioagricultural Science, Nagoya University, [2]

Bioscience and Biotechnology Center, Nagoya University, [3] Institute for Advanced Reserch, Nagoya University.

Recently, we showed that the sea urchin egg 350-kDa sperm-binding protein (SBP) binds to the major ganglioside in sperm lipid rafts in a sialic acid-dependent manner (Maehashi *et al*. (2003) *J. Biol. Chem*. **278**, 42050-42057). Interestingly, SBP shows highly homologous amino acid sequences to heat shock protein (Hsp) 70 family and the Hsp110 subfamily. The Hsp70 family is ubiquitous and highly conserved in prokaryotes and eukaryotes, and binds to posttranslationally unfolded or misfolded proteins in the course of quality control of proteins during their assembly and transport. It has been recently shown that the testis-specific Hsp70 localized on the sperm possesses the binding ability to 3-*O*-sulfated galactose-containing lipids. Furthermore, a constitutively expressed Hsc70 and a heat-inducible Hsp70, which share a high degree of sequence homology, have been shown to interact with liposomes containing phosphatidylserine. Our results together with others' ones led us to assume that the Hsp70 family has the ability to bind negatively charged glycolipids. To demonstrate this assumption, we assessed the binding ability of the mammalian Hsp70 family to various glycolipids and phospholipids. In this study, the binding activity of mouse heat-inducible Hsp70 to various glycolipids (GlcCer, GalCer, LacCer, GM3, GD3 and sulfatide) and various phospholipids (PI, PS, PC, SM) was assayed by the enzyme-linked immunosorbent assay-based method (ELISA). The Hsp70 preferentially bound gangliosides and sulfatide to neutral glycolipids. The binding of Hsp70 to the GD3-coated plastic surface was dependent on sialic acid, because the pretreatment of the GD3-coated surface with a sialidase greatly diminished the binding. Of those phospholipids tested, the highest binding of Hsp70 was observed for phosphatidylserine. These results demonstrate that Hsp70 recognizes negatively charged lipids including gangliosides, sulfatide, and phosphatidylserine.

(169) **Synthesis of Mini-lectins and their Carbohydrate Selectivity**

Hiroko Kawakami[1,2] and Kazunori Toma[1,2]
[1] The Noguchi Institute, [2] CREST, JST.

Lectins are useful tools to analyze the carbohydrate structure of glycoconjugates and to characterize various type of cells. They have, however, some practical problems: the number of commercially available lectins is limited; lectins are prone to be denatured; the carbohydrate specificity is predetermined. Therefore, designable synthetic compounds which recognize carbohydrate structures with lectin-like specificity are desired. Yamamoto et al. (*FEBS Lett.* 1991, 281, 258; *J. Biochem.* 1992, 111, 436) reported that some of endoproteinase-digested peptide fragments of a leguminous plant lectin bound to a cabohydrate-immobilized column. They showed that the peptide fragments retained the original carbohydrate-specificity of the lectin, and that the fragment corresponded to the carbohydrate and metal binding site of the lectin. Base on the knowledge, we synthesized "mini-lectins", lipid conjugates of the peptides that had the amino acid sequences of the carbohydrate-binding site of natural lectins. When the mini-lectin is coated on a hydrophobic surface, the lipid portion adheres to the surface, and the peptide portion is presented to the aqueous side. We chose the DTWPNTEWS peptide sequence of *Bauhinia purpurea* lectin, a galactose binding lectin, DSYFGKTYNPW of *Ulex europaeus* lectin II, a *N*-acetylglucosamine binding lectin, and DTWPNTDIG of concanavalin A, a mannose binding lectin. A 3,5-bis(dodecyloxy)benzamide derivative conveniently synthesized from methyl 3,5-dihydroxybenzoate was employed as the lipid portion. The peptide portion was extended from the terminal amino group of the lipid portion by the solution phase Fmoc strategy. The mini-lectins were immobilized on a hydrophobic cuvette of an IAsys SPR-biosensor, and the interaction with fetuin and asialofetuin was analyzed. The mini-lectin with DTWPNTEWS sequence showed more favorable binding with asialofetuin than fetuin. Thus, the mini-lectin was galactose-selective. The mini-lectin with DSYFGKTYNPW sequence did not bind to asialofetuin. However, the mini-lectin with DTWPNTDIG sequence exhibited the interaction with asialofetuin. Thus, there can be cases in which the carbohydrate specificity of a mini-lectin is different from the original lectin. This result may be ascribed to the similarity of sequence between the mannose-binding peptide and the galactose-binding peptide. It was also shown that the peptide portion of natural lectins did not recognize the hydroxyl group at C2 position of mannose through the inspection of the X-ray crystallographic structures of concanavalin A and other leguminous lectins.

(170) **Novel Artificial Lipid for the Construction of Neoglycolipid Probes**

Reiko Sato[1,2], Hiroko Kawakami[1,2] and Kazunori Toma[1,2]
[1] The Noguchi Institute, [2] CREAT, JST.

We previously synthesized neoglycolipid probes bearing 3,5-bis(dodecyloxy)benzamide as a lipid portion and a hexamethylene chain as a linker between the carbohydrate and the lipid portion. These neoglycolipids were shown to be useful as probes to analyze the interaction between lectins and carbohydrates, when the neoglycolipids were immobilized on a hydrophobic surface. Based on the same lipid structure, we also synthesized lipid analogues bearing various length of oligo-(ethylene glycol) (OEG) to be used as control compounds against the neoglycolipid probes, as it is known that the non-specific interaction between OEG and proteins is weak. These OEG-lipid analogues were prepared as simple esters by condensing 3,5-bis(dodecyloxy)benzoic acid and OEG. The OEG-lipid analogue mixed with the neoglycolipid was immobilized on a hydrophobic cuvette of a surface plasmon resonance biosensor, and the effect of OEG length on the interaction between lectins and carbohydrates was analyzed. In the case of concanavalin A and the mannose derivative, only the longest chain slightly disturbed the interaction. In the case of wheat germ agglutinin and the sialic acid derivatives, however, the longer OEG chain strongly inhibited the interaction. Here, we redesigned the lipid structure that could be used both as a lipid portion of neoglycolipids and also as a control compound. We employed OEG itself as a linker, and an amide linkage, in stead of the ester linkage, between OEG and 3,5-bis(dodecyloxy)benzoic acid. We synthesized four lipid analogues bearing tri- and hexa-(ethylene glycol) to investigate which length was suitable for our purpose. One or both terminals of tri- and hexa-(ethylene glycol) were successively tosylated, iodinated and phthalimidated. The OEG derivatives with one amino terminal or two amino terminals were obtained by the deprotection of the phtalimide group with hydrazine hydrate. The OEG derivatives were condensed with 3,5-bis(dodecyloxy)benzoic acid in good yields. The resultant lipid analogues were immobilized on a hydrophobic surface, and the interactions with bovine serum albumin (BSA) were analyzed by a surface plasmon resonance biosensor. The tri-(ethylene glycol) lipid of hydroxyl terminal slightly adsorbed BSA, but the hexa-(ethylene glycol) lipid of a hydroxyl terminal did not at all. Notwithstanding the OEG length, however, the other two lipid analogues with the amino terminal adsorbed a great amount of BSA. Therefore, we concluded that the novel lipid analogue with hexa-(ethylene glycol) must be useful as the basic structure for the construction of neoglycolipid probes. The introduction of carbohydrate moieties with reductive amination, which is often employed in the construction of neoglycolipid, may be problematic in view of the non-specific interaction.

(171) **Towards Defining Binding Specificities of Malaria Parasite *Plasmodium falciparum*-Infected Erythrocytes to Chondroitin Sulfate A and Hyaluronic Acid**

Wengang Chai[1], James G. Beeson[2], Timothy J. Byrne[2], Graham V. Brown[2] and Alexander M. Lawson[1]
[1] MRC Glycosciences Laboratory, Faculty of Medicine, Imperial College London, Northwick Park Hospital Campus, Harrow, Middlesex, UK , [2] Department of Medicine, University of Melbourne, Royal Melbourne Hospital, Victoria, Australia.

The severe pathology in *Plasmodium falciparum* malaria is largely due to adhesion of *P. falciparum*-infected erythrocytes (IEs) to endothelia and sequestration of IEs in the blood vessels of organs such as the brain, lung and placenta. Chondroitin sulfate A (CSA) [1] and, more recently, hyaluronic acid (HA) [2] have been identified as ligands for adhesion of the IEs that can lead to parasite accumulation in the placenta during pregnancy. We are examining the molecular basis of this adhesion [3] in order to better understand the cellular events of *P. falciparum*-IE interaction with CSA and HA. These are mediated principally by the multi-domain *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) of the var gene family, which is expressed at the surface of IEs and mediates adhesion to a range of receptors on vascular endothelial cells. This presentation is focused on direct binding studies with PfEMP1, isolated from cultures of IEs, and CSA or HA immobilized on solid matrices. For efficient immobilization, polysaccharides were biotinylated, and oligosaccharide fragments were converted to neoglycolipids. In order to preserve the functionality of the carbohydrate molecules, biotinylation was achieved through carboxyl groups of glucuronic acid residues at a ratio of 1 in every 50 carboxyl groups. Oligosaccharides were converted into neoglycolipid probes [4] by conjugation to an aminophospholipid at the reducing termini. The results

show that PfEMP1 binds to CSA and HA polysaccharides and to oligosaccharide fragments in a size-dependent manner. Optimum binding is observed to oligosaccharides of 12-14 monosaccharide residues or longer, with little binding to shorter fragments. By contrast, there is little or no binding to CSC polysaccharide or to CSC oligosaccharide probes. These results are consistent with our previous findings that the minimum chain length of CSA [5] or HA [6] required for effective inhibition of IE adhesion to immobilized receptors is 12-14 monosaccharide residues. The way is now open to defining the specificities of binding of the domain(s) of PfEMP1 to oligosaccharide sequences presented on host cell surfaces relevant to the pathogenesis of malarial disease. References: [1] SJ Rogerson, SC Chaiyaroj, K Ng, JC Reeder & GV Brown (1995) J Exp Med 182, 15-20; [2] JG Beeson, SJ Rogerson, BM Cooke, JC Reeder, W Chai, AM Lawson ME Molyneux & GV Brown (2000) Nat Med 6, 86-90; [3] W Chai, JG Beeson & AM Lawson (2002) J Biol Chem 277, 22438-2446; [4] W Chai, MS Stoll, C Galustian, AM Lawson & T Feizi (2003) Methods Enzymol 362, 160-195; [5] JG Beeson, W Chai, SJ Rogerson, AM Lawson & GV Brown (1998) Infect Immun 66, 3397-3402; [6] W Chai, JG Beeson, H Kogelberg, GV Brown & AM Lawson (2001) Infect Immun 69, 420-425.

(172) **Characterization of Human β 1,4GalNAc-T3 and -T4, Synthesizing LacdiNAc Structure.**

Takashi Sato[1], Masanori Gotoh[1], Yuzuru Ikehara[2], Katsue Kiyohara[1], Masae Tatematsu[2] and Hisashi Narimatsu[1]

[1] Glycogene function Team, Research Center for Glycoscience(RCG), National Institute of Advanced Industrial Science and Technology(AIST), [2] Division of Oncological Pathology, Aichi Cancer Center Research Institute.

LacdiNAc (GalNAcβ1,4GlcNAc-), a special carbohydrate structure in human *N*-glycans, has been found in pituitary glycoprotein hormones and certain glycoproteins, such as lutropin(LH), thyrotropin(TSH) and glycodelin(Gd). In the case of LH, sulfated lacdiNAc is recognized by carbohydrate receptor in hepatic endothelial cells and Kupffer cells, resulting rapid clearance of LH from blood. We have cloned and characterized two lacdiNAc synthases, β1,4-*N*-acetylgalactosaminyltransferases, β4GalNAc-T3 and -T4, which were typical type II membrane proteins containing a β1,4 glycosyltransferase motif at their C-terminus. Reconbinant β4GalNAc-T3 and -T4 expressed in human embryonic kidney cells transferred GalNAc toward GlcNAc with a β1,4 linkage on both *N*- and *O*-glycans of glycoproteins. Tissue distribution of two enzymes was determined by the real-time PCR method. β4GalNAc-T3 was highly expressed in stomach, pituitary gland and testis, while -T4 was expressed in ovary, whole brain and fetal tissues. We developed monoclonal antibodies against each enzyme for immunohistochemical analyses. In the immunostaining using anti-β4GalNAc-T3 monoclonal antibody, β4GalNAc-T3 was expressed in gastoric foveolar epithelia. We will discuss the tissue specific expression of two lacdiNAc synthases and lacdiNAc carrier proteins. This work was supported by the New Energy and Industrial Technology Development Organization(NEDO).

(173) **Investigations of Rainbow Trout Cytidine-5'-monophosphate (CMP-) Sialic Acid Synthetase Based on 1H, 31P and STD NMR Spectroscopy**.

Thomas Haselhorst[1], Anja M¸nster-K¸hnel[2], Anita Stolz[2], Melanie Oschlies[2], Joe Tiralongo[1], Rita Gerardy-Schahn[2] and Mark von Itzstein[1]

[1] Institute for Glycomics, Griffith University (Gold Coast Campus) PMB 50 Gold Coast Mail Centre, Qld., 9726, Australia, [2] Abteilung Zellul‰re Chemie, Medizinische Hochschule Hannover (MHH), Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany.

The recombinant rainbow trout CMP-sialic acid synthetase has been analysed by 1H, 31P and STD NMR spectroscopy methods. NMR spectroscopy is a powerful tool to investigate the formation of CMP-Kdn, CMP-Neu5Ac and CMP-Neu5Gc. Using 1H NMR spectroscopy relative rates have been determined for the transfer of CMP from CTP to Kdn, Neu5Ac and Neu5Gc. It was found that the Kdn was the preferred nonulosonic acid substrate and that the recombinant CMP-Kdn synthetase transfers CMP from CTP to Kdn with 2.4 and 3.2 times higher efficiency than to Neu5Ac and Neu5Gc, respectively. Saturation transfer difference (STD) NMR spectroscopy has been used to investigate the binding epitopes of CMP-Neu5Ac and CTP.

(174) **Expression of the Vacuolar H^+-ATPase 16 KDa Subunit Decreases β1 Integrin Cell Surface Levels by Preventing Calnexin-β1 Association, Resulting in Triton X-100 Insoluble Aggregation.**

Intaek Lee[1], Mhairi A. Skinner[2], Huabei Guo[1], Avinash Sujan[1] and Michael Pierce[1]

[1] Complex Carbohydrate Research Center and Dept. of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30605, USA, [2] The Department of Molecular Biology and Genetics, University of Guelph, Guelph, Ontario N1G 2W1, Canada.

Vacuolar H^+-ATPase (V-ATPase) functions as a vacuolar proton pump and is responsible for acidification of intracellular compartments such as the endoplasmic reticulum, Golgi, lysosomes and endosomes. Previous reports have demonstrated that 16 kDa (16K) subunit of V-ATPase, via one of its transmembrane domains, TMD4, strongly associates with β1 integrin, affecting β1 integrin N-linked glycosylation and inhibiting its function as a matrix adhesion receptor. Because of this dramatic inhibition of β1 integrin-mediated HEK-293 cell motility by 16K expression, we investigated the mechanism by which 16K was having this effect. Using HT1080 cells, whose α5β1 integrin-mediated adhesion to fibronectin has been extensively studied, expression of 16K also resulted in reduced cell spreading on fibronectin-coated substrates. A pulse-chase study of β1 integrin biosynthesis indicated that 16K expression down-regulated the level of the 110 kDa biosynthetic form of β1 integrin (premature form) and, consequently, the level of the 130 kDa form of β1 integrin (mature form). Further experiments showed that the normal levels of association between the premature β1 integrin form and calnexin were significantly decreased by expression of either 16K or one of its transmembrane segments, TMD4. Expression of 16K also resulted in a Triton-insoluble aggregation of an unusual 87 kDa form of β1 integrin. Interestingly, 16K co-immunoprecipitated with an antibody to calnexin, and vice versa, suggesting an association between calnexin and 16K during biosynthesis. A pulse-chase experiment, followed by sequential immunoprecipitation with anti-16K epitope and anti-calnexin antibodies, confirmed the calnexin-16K transient association. These results indicate that 16K expression inhibits β1 integrin surface expression and spreading on matrix by a novel mechanism that results in reduced levels of functional β1 integrin.

(175) **The Biological Evaluation of Disaccharides Derived from Heparin**

Rhona M. Duane[1], Alan O' Brien[2], Ciaran Lynch[1], Paul V. Murphy[2] and Kathy M. O' Boyle[1]

[1] Department of Pharmacology, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland, [2] Centre for Synthesis and Chemical Biology, Department of Chemistry, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland.

Up-regulated angiogenesis is characteristic in rheumatoid arthritis, diabetic retinopathy as well as during tumour growth and metastasis. Inhibitors of the angiogenic factor basic Fibroblast Growth Factor (bFGF) could potentially lead to new therapeutics. FGF elicits a cellular response by binding to the FGF receptor, an action that requires heparin or heparan sulfate proteoglycans (HSPGs). The disaccharide β-D-GlcA-(1→4)-α-D-GlcNAc-1→OMe and other small non-sulfated oligosaccharides with structural features found in heparin/heparan sulfate have been shown to bind bFGF and activate the FGF receptor in F32 cells. The aim of this study was to evaluate the effect of disaccharides, derived from heparin, on the growth of bovine aortic endothelial cells (BAECs) and human coronary aortic smooth muscle cells (HCASMCs) and also to determine their effects on the binding of heparin to bFGF. The dissacharides β-D-Glc-(1→4)-α--D-GlcNAc-1→OMe and 3-O-Benzyl-β-D-Glc-(1→4)-α--D-GlcNAc-1→OMe were synthesised by Dr. Paul Murphy and colleagues. Heparin-albumin was found to inhibit the survival of BAECs by 37% at a concentration of 10μg/ml after 24hrs, as determined using a methyl tetrazolium (MTT) assay. Using similar conditions involving serum, which favours optimal growth, the dissacharides showed no effect on cell growth. In the absence of serum, the dissacharides had no significant inhibitory effect on cell survival as compared with 33% inhibition seen in the presence of heparin. The effect of the compounds on the proliferation of BAECs was also studied by measuring the uptake of [^{3}H] thymidine. With conditions involving serum heparin-albumin (10% after 24hrs) was seen to be less potent than indicated by the MTT assay. The disaccharides confirmed results seen with the MTT assay having no effect on the proliferation of the cells. The dissacharides caused no inhibition of growth of smooth muscle cells in either media condition as determined by the MTT assay. ELISAs were used to investigate the binding of the disaccharides to bFGF in competition with heparin-albumin. Heparin-albumin was found to have an

IC50 of 46.2ng/ml and the maximum inhibition observed was 80%. However, the dissacharides had no effect on binding. Previous work, using different experimental conditions to those employed by our group, has shown that disaccharides were mimics of heparin/heparan sulfate. However, our evaluation of the dissacharides has shown that, under a variety of conditions, they are unable to mimic the effects of heparin. It would seem that synthetic fragments larger than disaccharides will be required if agents are to be developed from heparin that will have potential application in angiogenesis therapy. This work was supported by IRCSET, Bioresearch Ireland and the Programme for Research in Third-Level Institutions (PRTCI) administered by the HEA.

(176) **Incorporation and Detection of 9-Substituted Neu5Ac Derivatives on Cell Surface**

Shoufa Han, Brian E. Collins and James C. Paulson
Departments of Molecular Biology and Experimental Medicine, The Scripps Research Institute, San Diego, CA.

Neu5Ac, one of the most abundant species of sialic acid, usually resides in the terminal end of glycoconjugate glycans of glycolipids and glycoproteins. These glycans are involved in host-pathogen interactions, cell-cell adhesion, and cell signaling events. Incorporation of Neu5Ac derivatives with bioorthognal functionalities into living cells offers an invaluable tool to study the oligosaccharide-protein interactions.[1,2] 9-Substituted Neu5Ac derivatives, 9-azido-Neu5Ac (**1**), and 9-(4'-azidophenylacetamido)-Neu5Ac (**2**) were synthesized by efficient, high yield procedures. When present in the medium of BJA-B cells, a human B cell line, these derivatives were readily up-taken and expressed on the cell surface. Detection of **2** on the cell surface was evident but weak using the reported Staudinger reaction,[3] but was inefficient for detection of **1**. Model reactions reveal an unexpected intra-molecular rearrangement resulting in hydrolysis of the intermediate. Suprisingly, all sialic acid derivatives were readily detected on the cell surface by FACS analysis using the *Sambuccus nigra* agglutinin (SNA), which recognizes the Siaα2-6Gal linkage. Thus, SNA appears to fortuitously recognize sialic acids modified at the C-9 position.

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(177) **Differential Expression of the Galectin Repertoire from Zebrafish (*Danio rerio*) During Early Embryogenesis: Biochemical and Molecular Characterization**

Hafiz Ahmed[1], Shao J. Du[1], Richard A. Alvarez[2] and Gerardo R. Vasta[1]
[1] Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD, [2] Department of Biochemistry and Molecular Biology, Core H - Consortium for Functional Glycomics, Oklahoma City, OK.

Galectins are β-galactoside-binding animal lectins containing one (proto and chimera types) or two (tandem-repeat type) carbohydrate recognition domains with significant sequence similarity. They are known to modulate cell-cell and cell-extracellular matrix interactions, thereby participating in various biological processes such as development, growth, and apoptosis. However, the detailed mechanisms of their roles remain largely unknown. Zebrafish constitutes an ideal model for developmental studies because of their external fertilization, transparent embryos, rapid growth, and availability of a large collection of mutants. As a first step in addressing the biological roles in zebrafish embryogenesis, we identified and characterized three proto galectins (Drgal1-L1, Drgal1-L2, Drgal1-L3), one chimera galectin (Drgal3), and two tandem-repeat galectins (Drgal9-L1 and Drgal9-L2). Zebrafish galectins exhibited distinct patterns of temporal expression during embryo development. Drgal1-L2 is expressed post bud stage, and its expression is strikingly specific to the notochord. In contrast, Drgal1-L1 is expressed maternally in the oocytes. Drgal1-L3, Drgal3, and Drgal9-L1 are expressed both maternally and zygotically, ubiquitously in the adult tissues. The distinct temporal and spatial patterns of expression of members of the zebrafish galectin repertoire suggest that each may play distinct biological roles during early embryogenesis. Genomic structure of Drgal1-L2 revealed four exons, with the exon/intron boundaries conserved with the mammalian galectin-1. Interestingly, this gene also encodes an alternatively spliced form of Drgal1-L2 that lacks 8 amino acids near the carbohydrate-binding domain. The Drgal1-L2 gene has been mapped to the chromosome 3, at 5.66 centiRay from the marker fc23e05. An antisense 'loss-of-function' approach indicates that blocking Drgal1-L2 expression results in a developmental defect in muscle cell differentiation. Like mammalian proto type galectin-1, Drgal1-L2 preferentially binds to N-acetyllactosamine and LN structures on biantennary N-glycans. Determination of the carbohydrate specificities for the other galectins is underway and will be discussed in comparison to that of Drgal1-L2. [Supported by Grant RO1 GM58537-01 from the NIH to S.J.D. and Grant MCB-00-77928 from the NSF to G.R.V.]

(178) **Galectin-1 binds to CD43 bearing both core 1 and core 2 O-glycans**

Joseph D Hernandez[1], Julie Nguyen[1], Mabel Pang[1], Minoru Fukuda[2] and Linda G Baum[1]
[1] Department of Pathology and Laboratory Medicine, UCLA School of Medicine, 10833 LeConte Ave, Los Angeles, CA, 90025, [2] The Glycobiology Program, The Burnham Institute, La Jolla, CA 92037.

Cellular glycosylation controls T cell susceptibility to galectin-1 induced death. Thus, we must understand how changes in glycan structures affect galectin-1 interactions with specific T cell counterreceptors, such as CD43. CD43 is an abundant T cell mucin with over 80 O-glycans that facilitates galectin-1 induced T cell death. To investigate the contribution of CD43 to T cell binding of galectin-1, we compared galectin-1 binding to CD43+ and CD43- CEM T cells. CD43- cells bound substantially less galectin-1 than CD43+ T cells, demonstrating that CD43 captures a significant fraction of galectin-1 on the T cell surface. As CD43 can bear core 1 glycans with Galβ1,3GalNAc sequences that are low-affinity galectin-1 ligands, or core 2 O-glycans with Galβ1,4GlcNAc sequences that are high-affinity galectin-1 ligands, we asked if core 2 O-glycans were required for galectin-1 binding to CD43. Since both CD43 and CD45 can bear core 2 O-glycans, we compared galectin-1 binding to CD45- cells with and without core 2 O-glycans. Surprisingly, in the absence of CD45, galectin-1 binding to T cells was equivalent in the presence or absence of core 2 O-glycans. In addition, CD43 isolated from both core 2 O-glycan negative and positive cells bound immobilized galectin-1. Thus, while core 2 O-glycans contribute to galectin-1 binding to CD45, galectin-1 binding to CD43 does not require core 2 O-glycans. Thus, glycan structures important for galectin-1 binding and signaling are specific for individual glycoprotein counterreceptors, and presentation of multiple low-affinity ligands on a glycoprotein backbone may result in biologically relevant protein-carbohydrate interactions.

(179) **Molecular recognition by LARGE is essential for expression of functional dystroglycan**

Motoi Kanagawa[1], Fumiaki Saito[1], Stefan Kunz[2], Takako Yoshida-Moriguchi[1], Rita Barresi[1], Yvonne M. Kobayashi[1], John Muschler[3], Jan P. Dumanski[4], Daniel E. Michele[1], Michael B.A. Oldstone[2] and Kevin P. Campbell[1]
[1] Howard Hughes Medical Institute, Department of Physiology & Biophysics and Department of Neurology, The University of Iowa Roy J. & Lucille A. Carver College of Medicine, Iowa City, Iowa 52242, [2] The Scripps Research Institute, Division of Virology, Department of Neuropharmacology, La Jolla, California 92037, [3] California Pacific Medical Center Research Institute, San Francisco, California 94115, [4] Uppsala University, Department of Genetics & Pathology, Uppsala 751 85, Sweden.

Reduced ligand-binding activity of α-dystroglycan is associated with muscle and central nervous system pathogenesis in a growing number of muscular dystrophies. Post-translational processing of α-dystroglycan is generally accepted to be critical for the expression of functional dystroglycan. Here we show that both the N-terminal domain and a portion of the mucin-like domain of α-dystroglycan are essential for high-affinity laminin-receptor function. Post-translational modification of α-dystroglycan by glycosyltransferase, LARGE, occurs within the mucin-like domain, but the N-terminal domain interacts with LARGE defining an intracellular enzyme-substrate recognition motif necessary to initiate functional glycosylation. Gene replacement in dystroglycan-deficient muscle demonstrates that the dystroglycan C-terminal domain is sufficient only for dystrophin-glycoprotein complex assembly, but to prevent muscle degeneration the expression of a functional dystroglycan through LARGE recognition and glycosylation is required. Therefore, molecular recognition of dystroglycan by LARGE is a key determinant in the biosynthetic pathway to produce mature and functional dystroglycan.

(180) **Structural Analysis of the Sulfotransferase (3-OST-3) Involved in the Biosynthesis of an Entry Receptor for Herpes Simplex Virus 1**

Andrea F. Moon[1], Suzanne C. Edavettal[2], Joe M. Krahn[1], Eva M. Munoz[3], Masahiko Negishi[4], Robert J. Linhardt[3], Jian Liu[2] and Lars C. Pedersen[1]
[1] Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, [2] Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, [3] Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, NY 12180, [4] Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709.

Heparan sulfate (HS) plays essential roles in assisting herpes simplex virus infection and other biological processes. The biosynthesis of HS includes numerous specialized sulfotransferases that generate a variety of sulfated saccharide sequences, conferring the selectivity of biological functions of HS. We report a structural study of human HS 3-O-sulfotransferase isoform 3 (3-OST-3), a key sulfotransferase that transfers a sulfuryl group to a specific glucosamine in HS generating an entry receptor for herpes simplex virus 1. We have obtained the crystal structure of 3-OST-3 at 1.95 ≈ in a ternary complex with 3'-phosphoadenosine 5'-phosphate and a tetrasaccharide substrate. Mutational analyses were also performed on the residues involved in the binding of the substrate. Residues Q255 and K368 are essential for the sulfotransferase activity and lie within hydrogen bonding distances to the carboxyl and sulfo groups of the uronic acid unit. These residues participate in the substrate recognition of 3-OST-3. This structure provides atomic-level evidence for delineating the substrate recognition and catalytic mechanism for 3-OST-3.

(181) **Differential Expression and Function of the CD33-Related Siglecs Between Humans and Great Apes**

Nancy Hurtado-Ziola, Justin L. Sonnenburg and Ajit P. Varki
Glycobiology Research and Training Center and Departments of Medicine and Cellular & Molecular Medicine, University of California, San Diego.

The Siglecs (Sialic acid-binding Immunoglobulin Superfamily Lectins) are a recently discovered family of mammalian glycan-binding proteins that can recognize the terminal sialic acids of glycoconjugates. The CD33-Related Siglecs (CD33rSiglecs, namely Siglec-3, -5 through -11 and -XII in humans) are a subgroup of these molecules, which appear to be primarily expressed on cells of the innate immune system. All CD33rSiglecs are type-1 transmembrane proteins with an N-terminal sialic acid-recognizing V-set domain followed by a variable number of C-2 set domains, a transmembrane region and a cytosolic C-terminal domain that usually has two tyrosine-based signaling motifs, one of which conforms to a canonical negative regulatory ITIM motif. Although the true function of the CD33rSiglecs has yet to be discovered, available data are most consistent with an inhibitory signaling role in the innate immune response, mediated by recognition of host sialic acids as 'self'. CD33rSiglecs also interact with sialic acids on the same cell surface, typically resulting in 'masking' of their sialic acid-binding sites. Our recent studies have shown that humans and non-human primates have a similar clustered localization of CD33rSiglec genes, and that true orthologs can generally be identified within each cluster (Angata et al., PNAS, in press). However, humans no longer express CMP-sialic acid hydroxylase (CMAH) the enzyme required to generate one of the potential CD33rSiglec sialic acid ligands called N-glycolylneuraminic acid (Neu5Gc), from its precursor N-acetylneuraminic acid (Neu5Ac). This genetic change occurred after our last common ancestor with the great apes, and dramatically altered the 'Sialome' (the sialic acid makeup of a specific species) of humans when compared to that of the great apes. While great ape blood cells express about equal amounts of Neu5Ac and Neu5Gc, human blood cells express almost exclusively Neu5Ac. We also recently discovered that preferential recognition of Neu5Gc is the ancestral condition of most or all of the great ape (chimpanzee and gorilla) CD33rSiglecs (Sonnenburg JL, Altheide TK, Varki A. Glycobiology. 14:339-46, 2004). We therefore reasoned that the sudden and major change in the sialome of our hominid ancestors could have had a significant impact on the evolution, binding specificities and expression patterns of CD33rSiglecs. We have found that all human CD33rSiglecs can recognize both Neu5Ac and Neu5Gc. This presumably represents an evolutionarily-selected 'relaxation' in binding specificity that was necessary to 'remask' the Siglecs that had lost their Neu5Gc ligands. Also, there are differences in CD33rSiglec expression on monocytes and neutrophils between humans and great apes. While great ape cells often show multiple populations with different signal intensities, humans express a single bright peak for each Siglec in flow cytometry. Surprisingly, while humans showed almost no CD33rSiglec expression on lymphocytes, the great apes show a moderate to high expression of some Siglecs on these cells. Total leukocyte expression of some CD33rSiglecs also shows differences between humans and great apes. Overall, CD33rSiglecs appear to be rapidly evolving in primates, with an apparent further acceleration in humans. Additional studies are needed to define the mechanistic details and the implications for human health and disease.

(182) **Using Click Chemistry and ATRP to Create Glycopolymer-Virus Conjugates**

Eiton Kaltgrad, Krisnaswami S. Raja, Sayam Sen Gupta, Maria J. Gonzalez, Marianne Manchester and M.G. Finn
The Scripps Research Institute 10550 N. Torrey Pines Rd. La Jolla, CA 92037.

Cowpea Mosaic Virus (CPMV) is a unique and versatile molecule for use as a chemical scaffold. The icosahedral structure of the virus provides chemists with a large and regular array suitable for the attachment of small molecules and proteins. The presentation of these molecules in an ordered and consistent orientation can be achieved by chemically modifying addressable amino acids displayed identically on each subunit of the virus. Molecules presented on CPMV can achieve a high local concentration and an overall dilute concentration, making this an ideal platform for drug delivery or cell targeting. Carbohydrates have been functionalized to allow attachment to the virus surface for the purpose of presentation and polyvalent binding to ligands. Glycopolymers have also been prepared and similarly arrayed on virus scaffolds in order to target carbohydrate-specific receptors The synthesis and properties of these structures will be described, including measurements of their polyvalent binding in hemagglutination assays and their utility in shielding the virus coat protein from the mammalian immune response. This model system can be applied to a number of different carbohydrates and other small molecules for the purpose of cell targeting, imaging, and destruction.

(183) **Oligosaccharide Preferences of β 1,4-Galactosyltransferase-I: Crystal Structures and Enzyme Kinetics of the Met344His Mutant of Human β 1,4-Galactosyltransferase-I with the trisaccharides of *N*-Glycan moiety**

Velavan Ramasamy[1], Elizabeth E Boeggeman[1,2], Boopathy Ramakrishnan[1,2], Daniel M Ratner[3], Peter H Seeberger[3,4] and Pradman K Qasba[1]
[1] Structural Glycobiology Section, LECB, CCR, National Cancer Institute-Frederick MD 21702
[2] BRP, SAIC-Frederick. Frederick, MD 21702
[3] Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139.
[4] Laboratorium f,r Organische Chemie, ETH H˙nggerberg/HCI F 315, Wolfgang-Pauli-Strasse 10, CH-8093, Zurich, Switzerland .

The *N*-Glycans, composed of complex bi- or tri- or tetraantennary sugar units, play significant role in the cell-cell interactions and various biological processes. During their biosynthesis galactose is added to several different arms or branches of the asparagine-linked complex oligosaccharide chains. The β1,4-Galactosyltransferase-I (β 4Gal-T1) can transfer galactose residue to either of the two GlcNAc residues located at the non-reducing ends of a biantennary oligosaccharide chain. The two antennae differ only in the linkage to the core Man residue that is β-linked to chitobiose. Thus one antenna is attached to the carbon 3 (the 3 arm) and the other to the carbon 6 (the 6 arm) of β -linked mannose residue. To better understand the oligosaccharide preferences of the β 4Gal-T1 towards *N*-Glycans, we have carried out the crystallographic and enzyme kinetic studies of β 4Gal-T1 with various oligosaccharides of the *N*-Glycan moiety. In a previous study we showed that when residue Met344 in bovine β 4Gal-T1 is mutated to histidine, mutant Met344His in the presence of Mn^{2+} and UDP-hexanolamine, readily changes to the closed conformation that creates the acceptor binding site, thereby facilitating the structural analysis of the enzyme with various oligosaccharide acceptors. In the present study, the branch specificity of human β 4Gal-T1 was investigated with the human Met344His mutant that was crystallized in complex with UDP-hexanolamine, Mn^{2+}, and different trisaccharides. The following trisaccharides of the *N*-Glycan moiety were used: GlcNAc β 1-2Manα1-6Man (1-6-arm), GlcNAc β 1-2Manα1-2Man (1-2-arm), GlcNAc β 1-4Manα1-3Man (triantennary), GlcNAc β 1-2Manα1-3Man (1-3-arm), and

GlcNAc β 1-4GlcNAc β 1-4GlcNAc (chitotriose). Crystal data was collected at 2.0 ≈ for all the complexes, except for the complex with triantennary trisaccharide (1.9 ≈). In all the structures the human β 4Gal-T1-Met344His mutant was found to be in the closed conformation with the trisaccharides bound to it. The electron density for the core Man residue (the third residue from the non-reducing end) among the trisaccharides bound to β 4Gal-T1 was clearly observed in the 1-6-arm trisaccharide where the core mannose rests over Tyr286 making hydrophobic interactions. In contrast, the core Man residue of the 1-3-arm trisaccharide possesses vague electron density with partial occupancy. The enzyme kinetic analysis also indicated a preferential binding of the 1-6-arm trisaccharide to the mutant enzyme. The K_m of GlcNAc β 1-2Manα1-6Man is approximately 10-fold lower than the K_m for GlcNAc β 1-2Manα1-3Man and GlcNAc β 1-4Manα1-3Man, and 22-fold lower than the K_m for GlcNAc β 1-2Manα1-2Man and chitotriose. However, the catalytic activity of the Met344His mutant, in the presence of Mn^{2+}, with the trisaccharide GlcNAc β 1,2Manα1-6Man was inhibited above 0.1 mM concentrations, whereas much higher concentrations (1-2 mM) for the other four trisaccharides where needed to inhibit the catalytic activity. Since β 4Gal-T1 binds strongly to GlcNAc β 1-2Manα1-6Man and the turnover number, k_{cat}, is hardly reduced, the catalytic efficiency (k_{cat}/K_m) of the enzyme with this acceptor is high compared to the other trisaccharides used in this study. Structural and kinetics aspects of the β 4Gal-T1 with the trisaccharides will be presented in detail. Funded in part by DHHS #NO1-CO-12400.

(184) **A peptide Mimic of the Carboxylated Glycan Epitope Recognized by mAbGB3.1, an Anti-inflammatory Antibody**

Chui Sien Chan, Geetha Srikrishna and Hudson Freeze

Glycobiology Program, The Burnham Institute, La Jolla, California 92037.

Peptides have been shown to function as carbohydrate mimics in vivo and in vitro. We previously generated a mouse monoclonal antibody, mAbGB3.1, that reacts with a carboxylated glycan epitope on bovine lung oligosaccharides and on the Receptor for Advanced Glycation End Products (RAGE). This antibody blocks onset of inflammation in a mouse model of colitis and inhibits secretion of proinflammatory cytokines elicted by LPS in mouse macrophages. To isolate peptides that mimic the glycan epitope, we panned a T7 phage display library against mAbGB3.1. Ten candidate phage clones which were enriched after four rounds of panning were selected for further characterization. Using an ELISA, we identified the cyclic peptides, CDGVEDPEC, CDDGGVGLC, CDDGIGLQC and CDLLHMEDC as the strongest binders. None of the ten candidate clones bound to a control monoclonal antibody. Control phage clones isolated by binding to BSA showed no detectable binding to mAbGB3.1 or the control antibody. Synthetic cyclic CDGVEDPEC peptide bound to mAbGB3.1 with a Kd of 10 μM. This peptide inhibited binding of mAbGB3.1 to immunoaffinity purified RAGE and to acidic glycopeptides prepared from bovine lung. The peptide showed a moderate attenuation of LPS-elicited TNF-α activation in mouse macrophages soon after activation. We suggest that CDGVEDPEC may provide a functional mimic blocking inflammation via a mAbGB3.1-responsive pathway. (Supported by a grant from Johnson & Johnson, Inc.)

(185) **High Throughput Screening of Multiple Glycan Binding Proteins and Antibodies Using a Solid Phase Glycan Array - Deciphering the Carbohydrate Code**

Richard A Alvarez[1], Ola Blixt[2], Nahid Razi[2], Angela Lee[1], Thomas K Johnson[1], Anntette Fleshman[1], Ziad Kawar[1] and Richard D Cummings[1]

[1] Department of Biochemistry and Molecular Biology and Oklahoma Center for Medical Glycobiology, Oklahoma City, OK 73104, [2] Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037.

Encoded within the glycan structures elaborated on glycoproteins are rich binding motifs that define their binding specificity for recognition by lectins (glycan binding proteins - GBPs). These glycan-coded interactions promote cellular communication leading to multiple downstream events with major biological significance. Much of what we know about these carbohydrate-GBP interactions is based upon cell agglutination assays and inhibition studies using monosaccharides. In the past decade many investigators have demonstrated the feasibility of using glycans bound to solid phase surfaces as a high-throughput screening platform for assessing protein-carbohydrate interactions. The ability to interrogate numerous glycans with related structural motifs simultaneously offers a new dimension in deciphering the information coded within carbohydrate structures. Core H, in collaboration with Core D of the Consortium for Functional Glycomics funded by NIGMS, has deployed a robust glycan array that contains several hundred unique glycan structures. The array is available to investigators studying GBPs of interest to the NIGMS. The array includes glycans containing mono- and multimeric terminal structures, naturally derived N-linked glycopeptides, synthetic O-glycopeptides, and sulfated glycans. The complete list can be viewed under 'Resources' on the Consortium website at: **http://web.mit.edu/glycomics**. To date over 37 investigator projects from around the world, involving the screening of more than 150 GBPs, have been approved for analysis on the glycan array. These projects include the analysis of human, murine, bacterial, viral, marine and plant GBPs, and include known and novel GBPs from human and murine C-type, galectin, and Siglec families. In addition, numerous carbohydrate-specific antibodies have been characterized using the glycan array. To date, each GBP and antibody has demonstrated remarkably unique binding characteristics indicating that the glycan code is complex and specifically governs GBP binding and functional regulation. Examples of this diversity will be presented from representative lectin groups to illustrate the power of this approach and to encourage investigators to utilize this unique resource. This work funded by GM62116 NIGMS, NIH.

(186) **MBP-ligand Oligosaccharides Associated with an Anti-tumor Activity to Human Colon Cancer Cells**

Nobuko Kawasaki[1], Motoki Terada[2], Risa Inoue[2], Naoko Kadowaki[2], Kay-Hooi Khoo[3] and Toshisuke Kawasaki[2]

[1] Faculty of Medicine, Kyoto University, Koto606-8507, Japan, [2]]Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan, [3] Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan.

Mannan-binding protein (MBP) is a Ca^{2+}-dependent mammalian lectin specific for mannose, N-acetylglucosamine and fucose. The serum MBP activates complement through the lectin pathway and is an important component of innate immunity. Recently, we found that MBP had a potent growth inhibitory activity to a human colorectal carcinoma cell line *in vivo* by a complement-independent mechanism[1]. We previously reported that MBP recognizes and binds to Le^b and Le^a epitopes expressed on the cell surfaces of human colorectal carcinoma cell line, SW 1116. Å@In this study, we isolated and characterized the MBP-ligand oligosaccharides, which were assumed to be associated with and to trigger the anti-tumor activity of MBP. From the glycopeptides fraction prepared from the pronase digest of SW1116 cell lysates, MBP-ligand oligosaccharides were released by hydrazinolysis and pyridylaminated, and then isolated by an MBP affinity column. The majority of the MBP-ligand oligosaccharides bound to both affinity columns of anti-Le^b and anti-Le^a mAbs. The PA-derivatized MBP-ligand oligosaccharides were fractionated into the neutral and acidic fractions**.** Carbohydrate analysis indicated that MBP-ligand oligosaccharides contained high molecular size polylactosamine-type N-glycans with high fucose content. Endo-β-galactosidase digestion of the MBP-ligand oligosaccharides resulted in the decrease of their molecular sizes together with a marked reduction of the binding activity to the MBP column, indicating the presence of poly N-acetyllactosamine structures in the MBP-ligand oligosaccharides. The selective removal of fucose residues from MBP-ligand oligosaccharides by HF or TFA treatment resulted in almost complete loss of the binding activity to the MBP and the AAL columns. MS analysis of the MBP-ligand oligosaccharides after permethylation indicated that they consisted of a series of oligosaccharides with different molecular sizes of about 4,200 - 9,000 Da. The non-reducing ends of the chains were either Le^a or Le^b epitope followed by Le^a repeats. The PA-tagged reducing end moiety of the oligosaccharides was isolated by C18 cartridge from the endo-β-galactosidase digests of the PA-tagged ligand oligosaccharides. The smallest but most prominent PA-tagged core was a fucosylated trimannosyl core with 4 HexNAc attached.

Reference: 1) Ma, Y. et al.: *Proc. Natl. Acad. Sci. USA*, 96, 371-375, 1999

(187) **SPR Analysis of the Binding Property of Four Mollusc Lectins to Glycoproteins**

Yasuhiro Ozeki[1], Chihiro Iwahara[1], Naoko Masuda and Sarkar M. A. Kawsar[2]

[1] Dept. of Biochem., Facult. of Sci., Yokohama City University 22-2,Seto,Kanazawaku Yokohama,236-0027 JAPAN, [2] Dept. of Chem., Facult. of Sci., Univ. of Chittagong Chittagong, 4331 BANGLADESH.

Several kinds of lectins were purified from mollusc, such as bivalve, cattlefish, sea hare, and snail The biochemical properties of these lectins suggested to differ from representative animal lectin as galectin and C-type lectin. We purified these lectins and compared the saccharide binding affinity using a surface plasmon resonance (SPR) biosensor, BIAcore 3000. Fetuin, asialo-fetuin, bovine submaxillary mucin, asialo bovine submaxillary mucin, and asialo-agalacto-bovine submaxillary mucin were prepared and immobilized on the sensor chip as ligands. 17kDa GalNAc/Gal-binding lectin from mantle of mussel(M.edulis), a 63kDa Gal-binding lectin from mantle of cattlefish (T.pacificus), a 45kDa Gal-binding lectin from sea hare (A. kurodai) ovary, bullfrog (R.catesbeiana) egg galectin-1 were purified with affinity chromatography from row materials. A snail(H.pomatia) 79kDa type A GalNAc-binding lectin was purchased from SIGMA-Aldrich. Each 20microgram/ml lectin applied to an SPR biosensor and analysed their binding property and kinetics. Sensor grum as binding curve showing the molecular interaction between lectins and glycoprotein ligands appeared that All lectins interacted with these glycoproteins. However,the binding affinity was slight different. The sensor grum of sea hare lectin was similar to that of bullfrog galectin. Other lectins showed to be the slow dissociation pattern. This difference reflected it in a equilibrium constant. The mussel lectin, The dissociation constant (KD) value of the cattlefish lectin, the snail lectin revealed KD=e-8M to e-9M order, for sea hare lectin and bullfrog galectin having been KD=e-6M order. The binding property of mussel and cattlefish lectins were suggested to similar with snail lectin.

(188) **Characterization of VIPL**

Hideto Tozawa, Naoki Matsumoto and Kazuo Yamamoto
Dept. of Integrated Biosciences, Grad. School of Frontier Sciences, The Univ. of Tokyo.

Newly synthesized secretory glycoproteins undergo a quality control process that assures their proper folding, oligomerization, and maturation before exit from the endoplasmic reticulum (ER). Folded proteins are transported from the ER through the Golgi apparatus toward cell surface via transport vesicles. During this process, cargo receptors, such as ERGIC (ER-Golgi intermediate compartment)-53 and VIP36 (36-kD vesicular integral membrane protein), are thought to facilite transport and sorting of proteins from the ER to the plasma membrane. VIPL (VIP36-like) has been identified as a molecule having high homology with VIP36. VIPL is a type I membrane glycoprotein with similar domain organization as VIP36 and a ubiquitously expressed protein. It is assumed that VIPL is mainly localized in the ER, but the function is largely unsolved. The cDNA encoding hypothetical L-type lectin domain of VIPL was inserted into plasmid pET-3c and expressed in E. coli cells. We are now analyzing the interaction between recombinant soluble VIPL and several glycoproteins, such as porcine thyroglobulin, ovalbumin, and chicken IgY, by surface plasmon resonance methods using a BIAcore biocensor system. HA-tagged VIPL was also expressed in the mammalian cells and molecules interacting with VIPL in the cells are searched by using pull-down assay. By clarifying the molecules that associate with VIPL, we are aiming at the elucidation of the characteristics and function of VIPL in the cells.

(189) **Molecular Basis for Carbohydrate Recognition by Mouse Macrophage Galactose-type C-type Lectin (mMGL) 1 and 2**

Sarawut Oo-puthinan[1], Makoto Tsuiji[1], Kaori Denda-Nagai[1], Nicolai V. Bovin[2], Sachiko Nakamura[3], Jun Hirabayashi[3] and Tatsuro Irimura[1]
[1] 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan, [2] Mikluko Marpaya St. 16/10, Moscow 117-997, Russia, [3] 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan.

[Background and Aim] The MGLs are unique calcium-dependent lectins. They are type II membrane-bound glycoproteins having a single carbohydrate recognition domain (CRD) at their C-termini. They are implicated with uptake and presentation of Gal/GalNAc-bearing antigens by dedritic cells, regulation of trafficking of MGL-positive macrophages in delayed-type hypersensitivity, and antigen induced tissue remodeling. The mouse MGL have two genes which give rise to two highly homologous lectins, mMGL1 and mMGL2 (1). Difference in the amino acid sequences is almost exclusively seen in their cytoplasmic domains and CRDs. A solid-phase assay previously demonstrated sharp distinction in carbohydrate specificities between extracellular domain forms of these two lectins. mMGL1 has high affinity for Lewis^x conjugated biotinylated polyacrylamide polymers (Le^x-bio-PAA), whereas mMGL2 has the greatest preference for β-GalNAc- or α-GalNAc-bio-PAA (1). Molecular basis of differential carbohydrate specificities of mMGL1 and 2 should help to understand their biological functions and to provide information on the evolution of Le^x specificity from Gal-type C-type lectins. In the present report, site-directed mutagenesis was performed and the binding specificity was investigated by the surface plasmon resonance (SPR) and the frontal affinity chromatography (FAC). [Methods and Results] Site-directed mutagenesis of the CRDs of mMGL1 and mMGL2 was performed in a pair-wise manner to determine the residues responsible for distinct carbohydrate specificities at the amino acid level. Soluble CRD forms of recombinant mMGL1, mMGL2, and their mutants were prepared and purified by galactose-affinity chromatography. Binding of these CRD forms to immobilized Le^x- or β-GalNAc-bio-PAA were performed based on a SPR system. Pair-wise substitution of Val, Ala, Thr, or Phe at positions 61, 89, 111 or 125 in mMGL1 CRD, respectively, caused the considerable or total reduction in Le^x-bio-PAA binding, suggesting that these residues are involved in determining the specificity of mMGL1 CRD for Le^x. The results of mutations on mMGL2 CRD suggested that Leu, Arg, or Tyr at positions 61, 89, or 125 in mMGL2 CRD were implicated in the preference for GalNAc. The considerable reconstitutions of Le^x binding were observed with a limited number of relevant mutants of mMGL2 including R89A, S111T, and R89A/S111T. Only A89R mMGL1 showed an increase in GalNAc binding. This suggests that Ala89 and Thr111 on mMGL1 CRD and Arg89 on mMGL2 CRD play critical roles in determining the specificity for Le^x and for GalNAc, respectively. Additional binding assays based on the FAC were performed using various fluorescent oligosaccharides. From the retarded elution, relative affinity was determined. The FAC data were consistent with SPR binding data. Studies on the specificity of these mutated lectins with a variety of oligosaccharides indicated that there was no affinity to carbohydrate structure unrelated to Le^x, GalNAc, or Gal. Furthermore, molecular modeling illustrated the potential direct molecular interactions of Leu61, Arg89, and His109 in mMGL2 CRD with GalNAc and the indirect effect of Tyr125 on the preferential GalNAc binding. These findings suggested the fine mode of GalNAc recognition by mMGL2 CRD as compared with rat hepatic asialoglycoprotein receptor-1. (1) Tsuiji M. et al, J Biol Chem, 177:28892, 2002.

(190) **Structural and Functional Analyses of C-terminal Domain of the Earthworm Lectin EW29**

Atsushi Kuno[1], Tomohiro Ueda[2], Ryuichiro Suzuki[3], Zui Fujimoto[4], Tsunemi Hasegawa[3], Ken-ichi Kasai[2] and Jun Hirabayashi[1]
[1] Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8566, Japan, [2] Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Kanagawa 199-0195, Japan, [3] Department of Material and Biological Chemistry, Faculty of Science, Yamagata University, Yamagata 990-8560, Japan, [4] Department of Biochemistry, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan.

Carbohydrate-binding proteins defined as lectins exist ubiquitously in both animals and plants, but those from the phylum annelid are only rarely reported. Recently, genes for galactose-binding lectin, EW29 from the earthworm *Lumbricus terrestris*, were cloned [1]. According to the sequence analysis, EW29 was found to be composed of two homologous domains (14.5 kDa), *i.e.*, N-terminal and C-terminal domains to form a tandem-repeated type structure. Both domains contained triple-repeated QXW motifs, of which occurrence clearly defined EW29 as an R-type lectin [2]. Interestingly, two potential sugar-binding sites in the C-terminal domain are closely similar to that of the *Ricinus communis* toxin B chain (RTB). This observation suggests that the C-terminal domain of EW29 potentially has hemagglutination activity. Thus, it is of interest to compare details of the structures and functions between the C-terminal domain of EW29 and the other R-type lectins. In this study, we investigated for the first time for structural and functional aspects of the C-terminal domain of EW29. For this purpose, an appropriate expression vector for a truncated mutant comprising only the C-terminal domain (C-half) was constructed, and was used to transform *Escherichia coli*. The C-half protein was expressed and purified by affinity chromatography as described previously [1]. When hemagglutination activity was measured using rabbit erythrocytes, the C-half protein exerted reduced but significant activity by itself. This indicates that C-half retained at least two sugar-binding sites in agreement with the above observation. Next, the binding affinity of EW29 toward 49 PA-labeled oligosaccharides was determined using an automated frontal affinity chromatography (FAC) system and compared with that of the other galactose-specific R-type lectin, RCA120. As a result, it was

found that both of the lectins could recognize the terminal galactose-containing oligosaccharides with the same order of K_d values. However, detailed specificities as regards linkage isomerism and terminal modifications with sialic acid proved to be quite different between them. For this elucidation, structural study of the C-half on the basis of X-ray crystallography is now under way [3]. This work was supported in part by the New Energy and Industrial Technology Development Organization (NEDO), Japan and by the Program for Promotion of Basic Research Activities for Innovative Bioscience (BRAIN). References [1] Hirabayashi, J., Dutta, S. K. & Kasai, K. *J. Biol. Chem.* 273, 14450-14460 (1998). [2] Rutenber, E. & Robertus, J. D. *Proteins: Struct. Funct. Genet.* 10, 260-269 (1991). [3] Suzuki, R., et al. *Acta Cryst.* D, in press.

(191) **Immunoglobulin Binding Protein (BiP) coimmunoprecipitate with VIP36.**

Daisuke Nawa, Naoki Matsumoto and Kazuo Yamamoto
5-1-5 Kashiwanoha, Kashiwa, Chiba Prefecture, 277-8562, Japan.

The vesicular integral membrane protein, VIP36, is a type I membrane protein that shares significant homology to leguminous lectins. The ability to recognize high mannose-type glycans and the broad localization from the ER to the cis-Golgi indicates that VIP36 functions as a receptor that facilitates transport of various glycoproteins. However, little is known about ligands for VIP36. Also, how and where VIP36 catch and release target glycoproteins remains unknown. To elucidate the precise role of VIP36, we searched for proteins that interact with VIP36. A protein with a molecular weight of about 80,000 was co-immunopreipitated with VIP36 in the presence of crosslinker DSP. To prepare large amount of the crosslinked proteins, we performed a crosslinking experiment on large scale using HEK293 cells stably expressing VIP36. One of the proteins thus obtained was identified as immunoglobulin binding protein (BiP) by LC-MS/MS analysis. BiP was found to be a molecular chaperone in ER, and interacts transiently with non-native substrates to enhance their folding. However, BiP was not coimmunoprecipitatied with ERGIC-53 and VIPL, which are other cargo receptors with an L-type lectin domain. These observations suggest that BiP plays a pivotal role in the ER-to-Golgi transport of selected proteins mediated by VIP36.

(192) **Chondroitin Sulfate Chains on CD44 Negatively Regulate its Ability to Bind Hyaluronan**

Brian Ruffell and Pauline Johnson
Department of Microbiology and Immunology, #300-6174 University Boulevard,
University of British Columbia, Vancouver, B.C., V6T 1Z3, Canada.

CD44 is a widely expressed cell adhesion molecule that binds to hyaluronan, an extracellular matrix glycosaminoglycan.† The interaction of CD44 with hyaluronan is tightly regulated and has been implicated in inflammation and tumor metastasis.† CD44 can also bind to aggrecan and versican, chondroitin sulfate proteoglycans that form an important structural component of the extracellular matrix.† Furthermore, CD44 is itself a proteoglycan; the standard form of CD44 (CD44H) can be modified by chondroitin sulfate, while expression of one of the variably spliced exons (v3) can result in attachment of heparan sulfate and chondroitin sulfate.† Chondroitin sulfate addition to CD44 is necessary for binding to fibronectin and collagen XIV.† To determine if chondroitin sulfate addition could also influence binding of CD44 to hyaluronan, we first localized the chondroitin sulfate addition site to serine residue 180 in human CD44H.† CD44 and CD44 mutated at the serine residue (S180A) were expressed as soluble CD44-immunoglobulin fusion proteins in HEK293 cells and as full length proteins in murine L cells.† In both cases, sulfate incorporation by CD44 was reduced more than 70% by inhibiting glycosaminoglycan addition with *p*-nitrophenyl β-D-xylopyranoside or by enzymatic digestion with chondroitinase ABC.† Sulfate incorporation by S180A was also reduced more than 80%, indicating that the mutation abrogates chondroitin sulfate addition to CD44.† Furthermore, both soluble and cellular forms of S180A showed enhanced hyaluronan binding, as did CD44 treated to remove glycosaminoglycans or chondroitin sulfate.† Together, these data suggest that chondroitin sulfate modification of CD44H reduces its ability to bind hyaluronan.† Given that CD44 binds chondroitin sulfate proteoglycans via its hyaluronan binding region, we propose that chondroitin sulfate on CD44 negatively regulates hyaluronan binding by blocking this binding site.† This implies that factors regulating chondroitin sulfate synthesis may regulate the interaction of CD44 with hyaluronan and its associated functions.

(193) **Characterization of Carbohydrate-binding Activities of β-glucuronidase and α-amylase with Glycoligands Other Than Their Substrates**

Hiroko Matsushita-Oikawa[1], Mayumi Komatsu[1], Naoko Iida-Tanaka[3], Tetsuko Kanamori[2], Isamu Matsumoto[2], Nobuko Seno[2] and Haruko Ogawa[1]
[1] Course of Advanced Biosciences, Graduate School of Humanities and Sciences, Ochanomizu University, Tokyo, Japan, [2] Department of Chemistry, Faculty of Science, Ochanomizu University, Tokyo, Japan, [3] Department of Food Science, Otsuma University, Tokyo, Japan.

[Introduction] Novel carbohydrate-binding activities have been discovered for pancreatins, including α-amylase [(1-3)]. Whether the carbohydrate binding abilities that are characteristic of pancreatins extends to other enzymes is a key to elucidation of the biological significance of carbohydrate recognition. We found that a lysosomal enzyme, β-glucuronidase, which is an essential exoglycosidase in animals for degradation of glycosaminoglycans or conversion of steroid hormones for absorption, has carbohydrate-binding properties, and characterized to elucidate the difference between it and α-amylase. [Methods and results] Bovine liver β-glucuronidase was shown to bind best to a lactamyl Sepharose column with a pH optimum at pH 6-7, and it was separated most effectively from other contaminating enzymes, β-hexosaminidases and β-galactosidase, under the conditions of binding at pH 6 and elution at pH 5. On the other hand,β-glucuronidase bound to immobilized saccharo-1,4-lactone, a specific inhibitor, or 4-aminophenyl β-glucuronide, a substrate, but it was not separated from other enzymes because the coexisting enzymes bound and eluted concomitantly with β-glucuronidase. Although the elution from lactamyl-Sepharose was incomplete with 0.1 M lactose, 1-deoxy-4-*O*-β-D-galactopyranosyl-1-[(2- hydroxyethyl)amino]-D-glucitol, which was derivatized from lactose and 2-aminoethanol by reductive amination and has the same ligand structure as lactamyl Sepharose, could elute β-glucuronidase efficiently, suggesting that it bound to and was eluted from the column sugar-specifically. Further purification was performed by ion exchange HPLC on a DEAE-5PW column, and the purification was achieved by two-step chromatography. At neutral pH, purified β-glucuronidase bound to biotin-polymer (BP-) sugar probes of β-lactose and β-*N*-acetyllactosamine as well as β-Gal/β-GalNAc, in contrast to pancreatic enzymes that shared a high affinity toward α- mannose residues. β-Glucuronidase bound to asialofetuin better than to fetuin and asialoagalactofetuin, but not to transferrin, asialotransferrin and ovomucoid on solid-phase assay whereas α-amylase bound best to transferrin and to fetuin better than asialofetuin. In addition, β-glucuronidase interacted with lactosyl ceramide and polylactosaminoglycan of the band-3 glycoproteins but not to ceramide and deglycosylated band-3. Among the saccharides tested, only lactose activated β-glucuronidase noncompetitively at pH 5, indicating that the lactose-recognition site is different from the substrate-binding site. [Discussion] Both β-glucuronidase and α-amylase showed binding to carbohydrates other than their substrates, and the binding enhanced catalytic activities. Clear differences between the two enzymes were found in the binding specificities for saccharides and glycoproteins. Furthermore, β-glucuronidase also bound to lactosyl ceramide and polylactosaminoglycan in contrast to the ligands for α-amylase, which were mainly complex-type *N*-linked glycans. The optimum carbohydrate-binding was observed at neutral pH for β- glucuronidase. Considering the acidic pH in lysosomes, the carbohydrate-binding activity would be exhibited when β-glucuronidase is secreted extracellularly or in the route to early endosomes in the cell. On the other hand, the carbohydrate-binding activity of α-amylase is strong at the acidic pH found in zymogen granules and weakened at the slightly alkaline pH in the duodenum. Screening for endogenous ligands of these enzymes in the biosynthetic, secretion and circulation pathways is therefore necessary. 1) Matsushita, H., *et. al. J. Biol. Chem.*, 277(7), 4680-4686 (2002), 2) Ihara, Y., *et. al. Glycobiology*, 13(11), 878 (2003), 3) Takekawa, H., *et.al*. will present at US/Japan GLYCO, 2004.

(194) **Heparin-mediated Cellular Internalization of Hepatoma-derived Growth Factor: Role of the Conserved N-terminal HATH domain**

Shih-Che Sue[1], Wei-Tin Li[1], Yu-Chieh Lin[1], Jiun-Guo Yu[1], Shao-Chen Lee[2], Wen-guey Wu[2], Jeou-Yuan Chen[1] and Tai-huang Huang[1]
[1] Institute of Biomedical Sciences, Academia Sinica, Nankang, Taipei, Taiwan, [2] Institute of Bioinformatics and Structural Biology, College of Life Sciences, National Tsing Hua University, Hsinchu, Taiwan.

Human hepatoma-derived growth factor (hHDGF) is a heparin-binding protein that was implicated in tumorigenesis, vascular development, cell proliferation and transcriptional activation. NMR solution structure of hHDGF is reported here that it comprises an ordered N-terminal domain (HATH domain, ~ 100 a.a.) and a highly disordered C-terminal domain (~140 a. a.). The N-terminal HATH domain is proved to be responsible for specifically interacting with heparin that the disordered C-terminal region exhibits no heparin-binding ability but shows transformation activity to enhance cell saturation density and growth rate. Meanwhile we demonstrate that HATH domain along is capable to delivery fluorescence proteins, DsRED and GFP, into cytoplasm and nuclear in the different cell lines. HATH domain shows the ability to serve as a potential carrier for aiding protein internalization. The process is further examined in a heparin-expression-defective CHOK1 cell line to determine the importance of heparin. Noticeably, the defective expression of heparin in cell surface completely abolishes the internalization process of hHDGF, implying the critical role of binding to heparin in the case of hHDGF. These results prove that the N-terminal HATH region promotes the entry of protein into the cell through binding to heparin molecule, while the C-terminal region may be responsible for regulation of its activity Furthermore, the N-terminal HATH domain is identified to have monomeric and dimeric formations in physiologic condition, that dimeric form shows strong heparin-binding affinity of Kd ~ 10 nM, however monomer loses such strong binding-behavior. Biochemical studies are performed to characterize the heparin binding length and sequence for dimeric HATH domain. In final, a hypothetical mechanism is addressed here to allow us to prospect the correlation between dimeric property and heparin-mediated cell entry.

(195) **Molecular Cloning, Tissue Distribution and Construction of Recombinant Forms of the Mouse Galectin-4 and Galectin-6**

Petr Maly, Vladimira Markova, Zuzana Skopkova, Gabriela Jenikova, Jaroslava Kalaninova and Jitka Lachova

Laboratory of Molecular Glycobiology and Center for Molecular and Gene Biotechnology, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic.

Galectins, highly evolutionarily conserved beta-galactosides binding proteins and soluble-type (S-type) lectins, represent a structurally defined family of characterized and molecular defined members. Galectin-4 represents a proto-type molecule of monomer divalent members of the galectin subfamily, containing also galectin-6, -8, -9, -12. Recently it has been well documented that some of the family members, namely dimeric or monomeric monovalent galectin members, participate in important biological processes such as imune response, cell adhesion, signal transduction, development or forming the metastasis. To study biological role of the galectin-4 and galectin-6, we have recently cloned 15 kb genomic locus of the mouse galectin-4 from 129/Sv phage genomic library and partially sequenced this DNA. We also purified and sequenced mouse galectin-4 full length cDNA using Marathon-Ready cDNA library. Newly we isolated mouse galectin-6 full length cDNA and subcloned it into plasmid vector pCR3.1. A comparison of mouse galectin-4 and -6 sequences confirmed high structural similarity between both these galectins and helped us to find out primer pairs combinations for a specific amplification of both galectins. Using RT-PCR we found that galectin-4 as well as galectin-6 are expresed in all tested tissues including small intestine, colon, liver, kidney, heart and spleen. Galectin-4 was epressed also in cell lines BW-5147, P19 and 3T3 while galectin-6 expression was found only in P19 cells. These data indicate that galectin-4 and -6 are more broadly distributed and their function may not be limited to the alimentary tract. To verify a hypothesis that the N link region of the divalent galectins, spanning both carbohydrate-recognition domains (CRDs), plays an important role in ligand recognition and a high affinity binding, we constructed recombinant plasmids for the production of a recombinant form galectin-6 and the three forms of the mouse galectin-4, carrying normal two carbohydrate recognition domains or one of the both CRD domains only. Production of these modified molecules will allow, in addition, to map an expression profile of galectin ligands in tissues and mouse cell cultures and will contribute to the understanding of a crystal structure of the galectin molecules.

(196) **Role of the Human ST6GalNAc-I and ST6GalNAc-II in the Synthesis of the Cancer-associated Sialyl-Tn antigen**

Nuno T Marcos[1], Sandra Pinho[1], Catarina Grandela[1], Andrea Cruz[1], BÈnÈdicte Samyn-Petit[2], Anne Harduin-Lepers[2], Filipe Silva[1], Raquel Almeida[1], Vanessa Morais[3], Julia Costa[3], Jan Kihlberg[4], Henrik Clausen[5] and Celso A Reis[1]

[1] Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal, [2] UnitÈ de Glycobiologie Structurale et Fonctionnelle, UMR CNRS 8576, UniversitÈ de Science et Technologies de Lille, Villeneuve d'Ascq, France, [3] Instituto de Tecnologia Qu'mica e BiolÛgica (ITQB), Oeiras, Portugal, [4] Department of Organic Chemistry, UmeÅ University, UmeÅ, Sweden, [5] Department of Oral Diagnostics, School of Dentistry, University of Copenhagen, Denmark.

The abnormal expression of Sialyl-Tn antigen (Neu5Acα2,6GalNAc-α-O-Ser/Thr) is a common phenotypic change observed in cancer and is associated with cancer aggressiveness and poor prognosis. Sialyl-Tn is rarely observed in normal tissues but highly expressed in a huge variety of carcinomas, as well as in pre-malignant lesions of the gastrointestinal tract. Although the clinical significance of Sialyl-Tn has been extensively characterized, a functional role for this glycan in cancer cells remains to be elucidated. Among the α2,6-sialyltransferases identified that transfer Neu5Ac to GalNAc on glycoproteins, only ST6GalNAc-I and ST6GalNAc-II are candidate enzymes for the Sialyl-Tn biosynthesis. We have studied the substrate specificity of these human α2,6-sialyltransferases in vitro in order to evaluate their capability for Sialyl-Tn biosynthesis. Expression constructs were designed to encode soluble ST6GalNAc-I and ST6GalNAc-II and were expressed in insect cells, purified and concentrated. Substrate specificity of both enzymes was analyzed for the first time towards a panel of glycopeptides with well-defined carbohydrate structures and site of glycosylation, as well as glycoproteins and other synthetic glycoconjugates. Our results show that recombinant ST6GalNAc-I and ST6GalNAc-II efficiently mediated the transfer of sialic acid to O-linked oligosaccharides of fetuin and asialofetuin. Synthetic glyco-conjugates (glyco-PAA) and arylglycosides did not serve as acceptor substrates for ST6GalNAc-I and ST6GalNAc-II. These results suggest that the aglycone part in the substrate molecule is crucial for the activity and specificity of both enzymes. Detailed analysis of substrate specificity using GalNAc-glycosylated peptides showed that both ST6GalNAc-I and ST6GalNAc-II may act as Sialyl-Tn synthases. Our results also allowed us to conclude that, among the characteristics of the acceptor substrate that could influence the enzyme activity (such as number/density of acceptor GalNAc, length of the peptide), only the primary sequence of the glycopeptides appears to be determinant for the substrate specificity of the enzymes. We further established a gastric carcinoma cell line (MKN45) stably transfected with the full-length of either ST6GalNAc-I or ST6GalNAc-II and evaluated the carbohydrate antigens expression profile induced by each enzyme. Our observations show that ST6GalNAc-I acts preferentially on Tn antigen whereas the ST6GalNAc-II acts preferentially on T antigen. Our results show that ST6GalNAc-I is the major Sialyl-Tn synthase and strongly support the hypothesis that the expression of the Sialyl-Tn antigen in cancer cells is due to ST6GalNAc-I activity. Further studies using these transfected cell lines are currently in progress, and data on how different glycosylation profiles affect cell behaviour will also be presented. Supported by FCT (POCTI/CBO/44598/02) and FLAD.

(197) **b1,3-*N*-acetylglucosaminyltransferase-7(b3Gn-T7) Acts on Keratan Sulfate(KS)-related Glycans**

Akira Seko and Katsuko Yamashita

Sasaki Institute, Kanda-Surugadai 2-2, Chiyoda-ku, Tokyo 101-0062, Japan.

Keratan sulfate(KS) is one of the major glycosaminoglycans and consists of repeating unit of *N*-acetyllactosamine substituted with sulfate residues at the C-6 of Gal and GlcNAc. KS is present in cornea, brain, cartilage, and so on, attaching to specific proteins. It has been shown that KS has unique biological functions and is related with several diseases, but its biosynthetic pathway has not been fully understood. We previously showed that b1,4-galactosyltransferase-IV can act efficiently on KS-related oligosaccharides, and suggested its involvement in KS biosynthesis. Here, to assess which b3Gn-T(s) has the extension activity for KS-related oligosaccharides, we studied the substrate specificities of the members of b3Gn-T family. We constructed expression vectors containing b3Gn-T2 to -T7 and iGn-T cDNAs, and expressed them in COS-7 cells for enzyme sources. As KS-related glycans, we used Galb1-4(6S)GlcNAcb1-3Galb1-4(6S)GlcNAc(L2L2), Galb1-4(6S)GlcNAcb1-3(6S)Galb1-4(6S)GlcNAc(L2L4), and (6S)Galb1-4(6S)GlcNAcb1-3(6S)Galb1-4(6S)GlcNAc(L4L4) as acceptor substrates. As a result, among the seven Gn-Ts, only b3Gn-T7 had higher activities for L2L2 and L2L4 than that for

tetra-antennary oligosaccharide which was a good substrate for all the b3GnTs. In contrast to L2L2 and L2L4, L4L4 was not an acceptor substrate for b3Gn-T7 as well as the other b3GnTs, suggesting that 6-*O*-sulfation of the non-reducing terminal Gal inhibits the action of b3Gn-T7 and that attachment of b3-GlcNAc precedes 6-*O*-sulfation of Gal. These results suggested that b3Gn-T7 can be involved in the elongation of KS chains.
Seko and Yamashita, (2004) FEBS Lett., 556, 216-220.

(198) **Molecular Cloning and Characterization of a Human Multi-substrate Specific Nucleotide-sugar Transporter Homologous to *Drosophila fringe connection***

Shin Kamiyama[1], Takeshi Suda[1], Hisashi Narimatsu[2], Yoshifumi Jigami[2] and Shoko Nishihara[1,3].
[1] Laboratory of Cell Biology, Department of Bioinformatics, Faculty of Engineering, Soka University, [2] Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), [3] Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Agency (JST).

The translocation of nucleotide sugars from the cytosol into the lumen of the endoplasmic reticulum and Golgi apparatus is mediated by specific nucleotide-sugar transporters (NSTs). NSTs have been mentioned as possible crucial players in the synthesis of glycoconjugates. Recent studies have demonstrated that *Caenorhabditis elegans* gene of *sqv-7* and *Drosophila melanogaster* gene of *fringe connection* (*frc*) are implicated in growth-factor signaling during development through the regulation of proteoglycan synthesis. Although *sqv-7* and *frc* are considered to be orthologous to each other, it remains obscure which NSTs of humans have corresponding functions. In humans, UGTrel7 protein has a similar multi-substrate specificity to those of FRC and SQV-7; namely, UDP-glucuronic acid/ UDP-*N*-acetylgalactosamine. However, FRC is primarily localized to the Golgi apparatus, while UGTrel7 is localized to the ER.
In the present study, we identified a novel human NST gene, *hfrc1*, which is closely related to *Drosophila frc*, *C. elegans sqv-7*, and human *UGTrel7*. Hydropathy analysis and predictions of the transmembrane helices of the amino acid sequence revealed that HFRC1 are Type III transmembrane proteins with seven transmembrane domains. HFRC1 showed 54.3 %, 46.6 %, and 46.6% identities to UGTrel7, FRC, and SQV-7, respectively. HFRC1 was localized within the Golgi apparatus following its transient expression in HCT116 cells. In human tissues, *hfrc1* and *UGTrel7* displayed similar tissue distributions, each showing high levels of expression in the colon, stomach, lung, and leukocyte. However, the expression level of *hfrc1* transcripts was ten times that of *UGTrel7*.
HFRC1 had a multi-substrate specificity for UDP-*N*-acetylglucosamine (UDP-GlcNAc), UDP-glucose (UDP-Glc), and GDP-mannose (GDP-Man) with apparent *Km* values of 8.0, 2.1, and 0.14 μM, respectively, in the Golgi fraction of yeast cells expressing this gene. On the other hand, HFRC1 transported UDP-GlcNAc and UDP-Glc, but not GDP-Man, in the mammalian cells. These results indicate that HFRC1 transports UDP-GlcNAc and UDP-Glc in the mammalian cells, but transports GDP-Man alone in the yeast. Whether or not HFRC1 can act as a real GDP-Man transporter in mammalian cells needs to be evaluated in future investigations.
UDP-GlcNAc can be utilized by the enzymes that catalyze the elongation of heparan sulfate. The overexpression of the *hfrc1* gene in HCT116 cells modulated the cell-surface heparan sulfate expression status. These results suggest that HFRC1 takes part in the synthesis of heparan sulfate by regulating the level of UDP-GlcNAc. *Hfrc1* is expected to be involved in growth-factor signaling through the regulation of heparan sulfate synthesis in a way similar to that observed for *frc*.

(199) **Computational Model of Cytokine Receptor Regulation by Hexosamine and Golgi N-glycosylation Pathways**

Ken Lau[1], Emily A. Partridge[1], Judy Pawling[1], Cristina I. Silvescu[2], Vern Reinhold[2], Christopher W.V. Hogue[1] and James W. Dennis[1]
[1] Samuel Lunenfeld Research Institute, Mount Sinai Hospital, and University of Toronto, 600 University Ave, Toronto ON, [2] Chemistry, University of New Hampshire, Durham, NH, 03824, USA.

Glucose flux through the hexosamine pathway supplies UDP-GlcNAc for the synthesis of complex N-glycans. UDP-GlcNAc is the sugar donor for the branching N-acetylglucosaminyltransferases in the medial Golgi, and it is also required along with UDP-Gal for poly-N-acetyllactosamine (Galβ1-4GlcNAcβ1-3)$_n$ extension in the trans Golgi. Highly branched N-glycans, notably Mgat5-modified structures, are preferentially extended with poly-N-acetyllactosamine[1] and exhibit higher affinities for galectin-3[2]. We have recently demonstrated that poly-N-acetyllactosamine-containing N-glycans bind to multimerized galectin-3 at the cell surface, forming a molecular scaffold or lattice via glycoprotein crosslinking[3]. Hence, the avidity of this heterogeneous lattice is dependent on N-glycan biosynthesis, and it serves to regulate receptor clustering and delay constitutive endocytosis.
To better understand the relationships between the hexosamine pathway, Golgi N-glycan processing, and N-glycan multiplicity, we have developed a mathematic model of cytokine receptor regulation by the lattice. Assumptions required for the construction of the model are as follows: (1) Galectin-3 is not limiting for receptor binding. (2) The half-life of surface receptors at steady-state is dependent on the rate of constitutive endocytosis, the number of N-glycans per receptor, UDP-GlcNAc concentrations, and the cellular profile of GlcNAc-Ts activities in the Golgi. (3) The proportions of occupied N-X-S/T sites in the extracellular domains of glycoproteins is globally estimated to be ~70%[4]. (4) The molecular population of a single gene product is a Gaussian distribution of possible glycoforms with respect to their avidities for galectin-3. A threshold amount of total avidity per receptor is required for association with the galectin-3/glycoprotein lattice. (5) The peak levels of Erk and Smad2/3 activation by acute cytokine stimulation are proportional to the available surface receptors, and this is validated both experimentally and with an existing EGF signaling computational model[5].
Receptor kinases that promote growth, oncogenesis and cell metabolism (e.g. IGFR, EGFR, PDGFR, FGFR etc. designated as RK1) respond to hexosamine pathway flux (UDP-GlcNAc) in a graded manner, while receptors mediating morphogenesis (e.g. TGF-β designated as RK2) respond in a switch-like manner. We have examined the dynamics of the Golgi N-glycan processing pathway as the possible source of this ultrasensitivity. Multistep pathways with repetitive use of an effector substrate (UDP-GlcNAc) can display ultrasensitivity, especially when each sequential reaction declines in efficiency[6]. Indeed, the model predicts that N-Glycan branching and lactosamine extension as a function of increasing UDP-GlcNAc produce branched and poly-N-acetyllactosamine modified N-glycans with a Hill coefficient of ~4.
Finally, bioinformatics analysis shows that the RK1 class of receptors possesses a higher mean density of N-X-S/T sites compared to the RK2 class. The trend holds in *C. elegans* but not in *Drosophila*, where there is only one galectin gene, whereas worm and mammals have 10-12 galectins. The enzyme orthologue of Mgat5 is also missing in *Drosophila* but present in *C. elegans* (gly-2). The bioinformatics analysis supports a different utilization of glycosylation between receptors performing dissimilar functions, leading to differential regulation. The computational model may be useful in predicting the effects of hypomorphic alleles in the hexosamine and Golgi pathways that contribute to cancer, aging, diabetes and congenital disorder of glycosylation.

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(200) **Cloning, Expression, and Characterization of EDEM Homolog Proteins**

Yuko Natsuka[1,3], Kazuyoshi Hirao[1,3], Junji Nakamura[1,3], Shunji Natsuka[2,3], Nobuko Hosokawa[1,3] and Kazuhiro Nagata[1,3]
[1] Department of Molecular and Cellular Biology, Institute for Frontier Medical Sciences, Kyoto University, 53 Shogoinkawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan, [2] Department of chemistry, Graduate School of Science, Osaka University, 1-1 Machikaneyama-cho, Toyonaka, Osaka 560-0043, Japan, [3] CREST(Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation(JST).

The endoplasmic reticulum (ER), the place for folding and early process of maturation of newly synthesized proteins, has several mechanisms to discriminate correctly folded proteins from misfolded ones, assuring the quality of the protein products. Once the proteins synthesized in the ER are terminally misfolded, they are retrotranslocated from the ER and degraded by the cytoplasmic proteasome, a mechanism known as ER-associated degradation (ERAD). Most of the newly synthesized proteins accept N-

linked glycosylation in the ER, and the trimming of the N-glycans is known to play an important role for the quality control of glycoprotein biosynthesis, as well as ERAD. EDEM (ER-associated Degradation Enhancing α-Mannosidase-like protein) is a type II ER transmembrane protein, the expression of which is induced by ER stress through XBP1 pathway. EDEM has a glycosylhydrolase family 47 domain, which is conserved among class I α1,2-mannosidases, but exhibits no apparent enzymatic activity. Overexpression of EDEM enhances glycoprotein ERAD, suggesting its involvement in the recognition process of the proteins destined for degradation, although the exact mechanism remains to be clarified. A database search has been carried out to identify two new protein sequences homologous to EDEM, both have the glycosylhydrolase family 47 domain. The corresponding cDNAs have been cloned and transiently expressed in HEK293 cells as the influenza hemagglutinin epitope (HA)-tagged proteins, producing 110kD and 70kD protein products, respectively. Expression of the gene products has been examined by northern blotting and in situ hybridization, revealing the distinctive expression profiles of EDEM and the homologs. The results of further characterization of the proteins, as well as effects of their overexpression on glycoprotein ERAD will be discussed.

(201) **Structural-Chemical Characterization of Saccharomyces cerevisiae α-Glucosidase I**

Amirreza Faridmoayer and Christine Scaman
Food, Nutrition and Health, University of British Columbia, 6650 N.W. Marine Drive, Vancouver, B.C. Canada V6T 1Z4..

Processing α-glucosidase I regulates a key step in asparagine-linked glycoprotein biosynthesis by hydrolyzing the terminal α-(1,2) linked glucose from Glc3Man9GlcNAc2. Soluble α-glucosidase I was purified as a 98 kDa glycoprotein from *Saccharomyces cerevisiae* overexpressing *CWH41*. Histidine and tyrosine could be protected from modification by diethylpyrocarbonate and tetranitromethane, respectively, using the inhibitor 1-deoxynojirimycin (DNJM), suggesting that these residues are involved in the catalytic activity of the enzyme. DNJM could not prevent inactivation of enzyme treated with N-bromosuccinimide used to modify tryptophan residues. Catalytically active polypeptides formed by endogenous and trypsin hydrolysis were inhibited by DNJM. A 37 kDa polypeptide was isolated as the smallest active fragment from both digests using carboxypentyl-DNJM based resin. N-terminal sequencing demonstrated that the hydrolysis site was between Arg521 and Thr522 for endogenous proteolysis and Lys524 and Phe525 for the trypsin-generated peptide. Treatment of the 37 kDa active polypeptide with N-glycosidase F showed that it is not *N*-glycosylated. It is suggested that histidine and tyrosine residues are involved in the binding site of the yeast enzyme, while this function likely has been replaced by cysteine and tryptophan in the mammalian enzyme ortholog. The catalytic domain of α-glucosidase I, which is located at the C-terminal of protein is resistant to hydrolysis and remains fully functional after cleavage.

(202) **Functional Domains of Processing α-Glucosidase I of Saccharomyces cerevisiae**

Amirreza Faridmoayer and Christine Scaman
Food, Nutrition and Health, University of British Columbia, 6650 N.W. Marine Drive, Vancouver, B.C. Canada V6T 1Z4..

Processing α-glucosidase I, encoded by *CWH41*, is a type II membrane bound *N*-linked glycoprotein located in the endoplasmic reticulum (ER). α-Glucosidase I trims the outermost glucose residue from the newly assembled $Glc_{\alpha(1\text{-}2)}Glc_2Man_9GlcNAc2$ with net inversion of configuration, and therefore is a key control point moderating *N*-linked glycosylation in the cell. A soluble form of α-glucosidase I is produced by endogenous cleavage between A_{24} and T_{25}, and can be further hydrolyzed between F_{525}-M_{526} to produce a highly active 37 kDa polypeptide. Therefore, truncated forms of α-glucosidase I F_{34}-F_{833} and M_{526}-F_{833} were cloned and over-expressed in *Saccharomyces cerevisiae* AH22 as C-terminus 6◊His tagged polypeptides to investigate the function of these enzyme domains.

F_{34}-F_{833} polypeptide was over-expressed and purified to 95% homogeneity by a series of anion exchange and metal affinity chromatographies. The purified F_{34}-F_{833} polypeptide had a specific activity of 3394 U/mg of protein, comparable with the specific activity of soluble α-glucosidase I isolated from over-expression of *CWH41* (3130 U/mg of protein). The polypeptide was not *N*-glycosylated, as treatment with N-glycosidase F did not reduce molecular weight.

The polypeptide, M_{526}-F_{833}, was over-expressed but was found to be inactive against the synthetic trisaccharideα-D-$Glc_{(1,2)}$α-D-$Glc_{(1,3)}$α-D-Glc-$O(CH_2)_8COOCH_3$. Interestingly, the same 37 kDa polypeptide produced by trypsin hydrolysis of either soluble Cwh41p or polypeptide (F_{34}-F_{833}) was active, with a higher turnover compared to intact forms. This result suggests that the region from F_{34} to F_{525} is required for the catalytic domain to obtain an active conformation during translation, but once formed, the catalytic domain is intrinsically stable. As well, glycosylation of the enzyme is not required for proper folding and therefore it does not affect enzyme activity. The lack of *N*-linked glycosylation of F_{34}-F_{833} polypeptide indicates that the N-terminal domain of Cwh41p alone, may contain the ER retention signal.

(203) ***Drosophila* Orthologues of Human Alzheimer's β-secretase Induce the Secretion of a Golgi-resident Transferase**

Norihiro Kotani[1,4], Shinobu Kitazume[1], Keisuke Kamimura[2], Satomi Takeo[2], Toshiro Aigaki[2], Hiroshi Nakato[3] and Yasuhiro Hashimoto[1]
[1] Glyco-chain Functions Laboratory, Frontier Research System, The Institute of Physical and Chemical Research, RIKEN, [2] Department of Biological Sciences, Tokyo Metropolitan University, [3] Department of Genetics, Cell Biology and Development, The University of Minnesota, [4] Department of Biochemistry, Osaka University Graduate School of Medicine.

Å@Alzheimer's β-secretase (BACE1) is identified as a pepsin-like membrane-bound aspartic protease that cleaves amyloid precursor protein (APP). APP is cleaved by BACE1 to produce a soluble NH_2-terminal fragment and a membrane-bound COOH-terminal fragment. The latter fragment is further cleaved by γ-secretase to result in the production of neurotoxic Aβ peptide. Aβ peptide then deposits in the brain, and the deposits are a hallmark of the pathology of Alzheimer's disease. Therefore BACE1 plays a crucial role in the initiation of Aβ formation and development of Alzheimer's disease.

Å@We previously found that BACE1 also cleaves a glycosyltransferase, α2,6-sialyltransferase, as a physiological substrate (1, 2). In the present study, we tried to identify BACE1 orthologues in *D. melanogaster* by BLAST homology search. We found the two candidates as orthologues of human BACE1, and isolated their cDNAs. The proteins encoded by the cDNAs were designated as DASP-(Drosophila ASpartic Protease)-1 and DASP2, which had 59% and 50% similarity to human BACE1, respectively. Each protein contained a pair of active site motifs (Asp/Thr or Ser/Gly), which is a common characteristic of aspartic proteases including BACE1. Although DASP1 and DASP2 did not contain an apparent transmembrane domain, the proteases overexpressed in COS cells were localized in the Golgi area. Some of the DASP1 overexpressed in S2 cells was secreted, but DASP2 was not. DASP1 transcripts were expressed in the head of fruitflies, whereas DASP2 transcripts were mainly in the body. When either DASP1 or DASP2 was coexpressed together with a certain Golgi-resident transferase derived from *Drosophila*, the proteases enhanced the secretion of the transferase from the cells. In conclusion, we have identified two functional *Drosophila* orthologues of BACE1, DASP1 and DASP2, which induce the cleavage and secretion of a certain Golgi-resident transferase. Gain-of-function or loss-of-function experiments for DASPs using *Drosophila* as a model will be a promising way to address the biological relevance of the protease *in vivo*.

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(2) Kitazume et al.: ***J Biol Chem***, 278: 14865-14871 (2003)

(204) **Cysteine Residues of *Aspergillus saitoi* 1,2-alpha-mannosidase Contribute to Thermostability**

Yota Tatara[1], Byung Rho Lee[1], Takashi Yoshida[2] and Eiji Ichishima[1]
[1] Laboratory of Molecular Enzymology, Graduate School of Engineering, Soka University, Hachioji, Tokyo, 192-8577, Japan, [2] Department of Biotechnology, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036-8561, Japan.

A. saito 1,2-alpha-mannosidase(1) contains three cysteine residues. We showed that Cys^{334} and Cys^{363} formed a disulfide bond, and Cys^{443} contained a free thiol group. To investigate the role of the cysteine residues, each cysteine residue was substituted to alanine. The cysteine residues were not required for the activity. A mutation at each cysteine residue greatly destabilized the enzyme. Cys^{334} and Cys^{363} form a disulfide bond to stabilize the enzyme and Cys^{443} also plays an important role in thermostability,

although it is not involved in a disulfide bond. (1) Tatara, Y. *et al.* (2003) *J. Biol. Chem.* **278**, 25289-25294

(205) **The ldb Phenotype of Saccharomyces cerevisiae Reflects a Reduction in Mannosylphosphate Groups Attached to the Outer Chain of N-linked Oligosaccharides**

Isaac Corbacho, Isabel Olivero and Luis M. Hernandez
Department of Microbiology. University of Extremadura. 06071 Badajoz. Spain..

It is well established that mannoprotein-linked oligosaccharides in Saccharomyces cerevisiae are phosphorylated by the transfer of mannosylphosphate groups to the position 6 of specific mannoses in the growing oligosaccharide. N-linked residues bear mannosyl-phosphate residues on both the inner core and outer chain (1), while some O-linked residues also bear one such group on the second mannose counting from the reducing end of the molecule (2). The low dye binding phenotype (ldb) in Saccharomyces cerevisiae has previously been associated with a reduction in the incorporation of phosphate groups into the mannoprotein-linked oligosaccharides (3). In this work we have analyzed the contribution of the phosphate groups located in the inner core and the outer chain of the N-linked oligosaccharides, as well as in the O-linked residues, to the expression of the ldb phenotype. We have recently performed a genome-wide search for non-essential genes whose deletion resulted in the expression of the ldb phenotype. The results with genes involved in synthesis of N- and O-linked oligosaccharides were as follows: - Strains deleted in genes encoding subunits of mannan polymerase I (VAN1 and MNN9) showed a severe phenotype. - Strains deleted in genes encoding subunits of mannan polymerase II (HOC1, MNN8, MNN9, MNN10, MNN11) showed a severe phenotype. - Strains deleted in genes involved in branching of the outer chain (MNN2 and MNN5) showed a severe phenotype. - A strain deleted in ALG12, which is involved in synthesis of one of the acceptor sequences for mannosylphosphate in the inner core, was not selected. - Strains deleted in genes involved in synthesis of the O-linked oligosaccharides (PMT1-6, KTR1, KTR3, KRE2) were not selected. Conclusions: 1. The lack of mannosylphosphate groups located on the outer chain of N-linked oligosaccharides results in a severe ldb phenotype. 2. The lack of mannosylphosphate groups located on the inner core of N-linked oligosaccharides does not modify the dye binding behaviour of the cell, when the outer chain is present. 3. Defects in the synthesis of O-linked residues and the concomitant reduction in mannosylphosphate groups attached to them do not modify the dye binding behaviour of the cell as long as the outer chain of N-linked oligosaccharides is present. 4. The contribution of the KRE2/MNT1 gene family to the branching of the outer chain is undetectable and totally overwhelmed by MNN2 and MNN5. As stated above, the strains deleted in MNN2 and MNN5 showed a severe phenotype, while the strains deleted in KRE2, KTR1, or KTR3 showed a dye binding behaviour indistinguishable from that shown by the wild type. References: (1) Hern·ndez, L.M., et al., (1989) J Biol Chem 264, 13648-13659. (2) Nakayama, K. et al., (1998) Biochim Biophys Acta 1425, 255-62. (3) MaÒas, P. et al., (1997) Glycobiology 7, 487-497. This work was supported by grant 2PR03A080 from Junta de Extremadura.

(206) **Analysis of the VPS74 gene of Saccharomyces cerevisiae Using DNA Microarrays**

Isaac Corbacho[1], Isabel Olivero[1], Stefan Hohmann[2] and Luis M. Hernandez[1]
[1] Department of Microbiology. University of Extremadura. 06071 Badajoz. Spain.,
[2] Department of Cell and Molecular Biology/Microbiology, G^teborg University, Sweden.

The VPS74 gene of Saccharomyces cerevisiae was initially described as involved in vacuolar protein sorting. It was selected in a genomic screen for genes whose deletion resulted in an alteration in transport of carboxypeptidase Y to the vacuole and a concomitant secretion of the protease into the culture medium (1). We have recently performed a genome-wide search for Saccharomyces cerevisiae non-essential genes involved in mannosyl phosphorylation of mannoprotein-linked oligosaccharides, selecting for genes whose deletion resulted in a decrease in the affinity for the dye alcian blue (ldb phenotype). The strain bearing a deletion in VPS74 was also selected in our screen. It showed a severe ldb phenotype and a significant defect in N-linked glycosylation as reflected in the reduction in size of the secreted invertase. The electrophoretic mobility of the secreted invertase agreed with a significant reduction in size of the outer chain of N-linked oligosaccharides, a reduction which certainly contributes to the ldb phenotype. Since the precise function of this gene has yet to be defined, we have started a functional analysis of VPS74 taking advantage of microarray technology which allows the study of genome-wide changes in gene expression in different strains or in the same strain under different environmental conditions. We analysed the gene-expression pattern of vps74-delta and compared it to that of the wild-type under standard growth conditions (YEPD, 28∫C). The cells were harvested in the exponential phase of growth, and processed following standard previously published methods. Every experiment was repeated at least 4 times, considering the mean of four repetitions. Although we found no extremely high alterations in the expression levels of most genes in the deleted strain, we selected a list of 31 genes with statistically significant expression changes of at least two-fold compared to the wild-type. Of them, 27 were down-regulated and 4 were up-regulated. The down-regulated genes belonged to different functional families, an example being VTC3, related to the phosphate metabolism family, which encodes a polyphosphate synthetase located in the vacuolar membrane. The decrease in polyphosphate accumulation in the vacuole could be one possible explanation for the ldb phenotype. Other genes encode proteins involved in aerobic respiration, carbohydrate metabolism, response to nutrient starvation, signal transduction, etc. We also found several uncharacterized ORFs: YBR139W, which encodes a protein located in the vacuole with carboxypeptidase C activity, and YFL054C, which encodes a membrane protein involved in water/glycerol transport. In a parallel experiment we found that the vps74-delta strain showed a defect in using glycerol as energy source when compared to the wild type. Five more uncharacterized ORFs are totally unknown. The results point to a role for Vps74p in several processes involved in obtaining energy. The observed defects in protein transport through the Golgi or to the vacuole are probably indirect effects. (1) Bonangelino, C.J. et al., (2002). Mol Biol Cell 13, 2486-2501. The microarray experiments were done in the Department of Cell and Molecular Biology/Microbiology, G^teborg University, Sweden. Part of this work was supported by grant 2PR03A080 from the Junta de Extremadura.

(207) **Structural Analysis of Human α1,6-Fucosyltransferase**

Hideyuki Ihara[1], Sachiko Toma[2], Yoshitaka Ikeda[3], Jianguo Gu[1], Motoko Takahashi[1], Eiji Miyoshi[1], Tomitake Tsukihara[4], Atsushi Nakagawa[4] and Naoyuki Taniguchi[1]
[1] Department of Biochemistry, Osaka University Medical School / Graduated School of Medicine. The 21st Century Center of Excellence Program., [2] Graduate School of Pharmaceutical Sciences, Kumamoto University, [3] Department of Biomolecular Sciences, Saga University Medical School, [4] Institute for Protein Research, Osaka University.

Mammalian α1,6-Fucosyltransferase (FUT8) is a typical type II membrane protein as a Golgi apparatus resident glycosyltransferase. This enzyme catalyses the transfer of fucose residue from the donor substrate, GDP-β-L-fucose, to a innermost GlcNAc of asparagine linked oligosaccharide (*N*-glycan) via an α1,6 linkage with the inversion of the anomeric configuration of C-1 position of fucose residue. We have reported that the high expression of FUT8 and the resulting elevation of α1,6-fucosylation were observed in hepatomas and ovarian cancer. Recently, it has been reported that the lack of α1,6-fucosylation of *N*-glycan-glycan on IgG1 increased activity by up to 50-100 fold antibody-dependent cellular cytotoxicity (ADCC). In addition, although there was no apparent anomaly on embryonic development in FUT8 knockout mice, FUT8-null mice manifest severe growth retardation and die within 4 weeks, suggesting that α1,6-fucosylation plays crucial in the regulation of proliferation and differentiation after birth. However, the catalytic mechanism and whole structure of FUT8 remains unclear. Previously. FUT8 has been shown that two arginine residues (Arg-365 and Arg-366) of human FUT8 in the short region conserved in FUT8, α1,2-fucosyltransferase and bacterial α1,6-FucT (also known as NodZ) have the important role for the donor substrate binding. Recently, FUT8 has been reported to have three short regions strongly conserved among FUT8, mammalian α1,2-fucosyltransferase and protein *O*-fucosyltransferase. In this study, we show the crystal structure of recombinant human FUT8. Recombinant enzyme was designed as secretory form by deliting transmembrane region and tagging with poly histidine at C-terminal site. The enzyme was expressed using baculovirus / insect cell expression system. Overall structure of FUT8 revealed a novel form distinguished from the structures of other glycosyltransferases, which have the DXD motif for binding divalent cation. At N-terminal region, the featured helix structure was observed. In addition, FUT8 has a SH3-like

domain at C-terminal in spite of a Golgi resident glycosyltransferase. Taking together, these results suggest that FUT8 may be a unique glycosyltransferase having feature modules on the aspect of structural basis.

(208) **Comprehensive Characterization of Human ppGaNTases**

Kouichi Tachibana, Yeon-Dae Kwon and Hisashi Narimatsu
Research Center for Glycoscience, the National Institute of Advanced Industrial Science and
Technology 1-1-1, Umezono, Central 2, OSL-C2, Tsukuba, Ibaraki, zip 305-8568, Japan.

O-Glycosylation is one of the major posttranlational modifications of cell surface and secreted proteins. The major O-Glycans on modified proteins start withÅ@GalNAc(α1-O)Ser/Thr, and such O-Glycans appear to play important roles in protein and cellular functions, such as cell adhesion and differentiation. However, the O-Glycan research has been considerably delayed compared to the N-Glycan research, because the O-Glycosylated proteins and the O-Glycosylated sites are mostly unclarified, and also because few methods are available to identify the O-Glycans. Here we show basically all human UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases (ppGaNTases), which are the glycosyltransferases that attach GalNAc to the Ser/Thr residues within the peptide backbones of proteins. At this time, there are 14 human ppGaNTases published, 5 of them from us. In addition, we cloned 4 more novel human ppGaNTases with conventional ppGaNTase structure. We have characterized these ppGaNTases by real-time PCR for tissue distribution and by in vitro enzyme assay with synthetic peptides for catalytic activity. These 18 ppGaNTases show difference in expression and catalytic activity, indicating that these ppGaNTases generate the diversity of the O-Glycosylation. The information of all human ppGaNTases contributes the further understanding of O-Glycosylation and is a helpful tool of O-Glycan research. This work was supported by the New Energy and Industrial Technology Development Organization(NEDO).

(209) **Potential Interaction Between N-acetylglucosamine Kinase and Different Proteins Using a Yeast Two Hybrid Screen**

Markus Berger, Felista Tansi, Werner Reutter and Stephan Hinderlich
CUB - Campus Benjamin Franklin, Institut f¸r Molekularbiologie und Biochemie, Arnimallee 22, 14195 Berlin-Dahlem.

N-Acetylglucosamine (GlcNAc) is a major component of complex carbohydrates. It is found in N-glycans and O-glycans as well as in glycolipids. Furthermore, in cytosolic and nuclear proteins GlcNAc is linked as a monosaccharide to serine-and threonine-residues, and may be a counterpart to O-phosphorylation at these residues. GlcNAc kinase converts endogenous GlcNAc from lysosomal degradation or nutritional sources into GlcNAc 6-phosphate. GlcNAc 6-phosphate then enters the pathway leading to the formation of UDP-GlcNAc, which serves as a substrate of the GlcNAc-transferases in oligosaccharide biosynthesis. In organs with high requirements of energy, like brain, intestinal tract and testis, only the salvage pathway can serve the high amounts of GlcNAc for the biosynthesis of UDP-GlcNAc. The oligosaccharide patterns in various cell types need different combinations of enzymes for these biosynthesis. A yeast two-hybrid screen, performed with GlcNAc kinase suggested interaction between functional different proteins.

(210) **Characterization of a Novel Human Core-specific lysosomal α1,6-mannosidase Involved in Glycan Catabolism**

Chaeho Park, Leslie Stanton, Robert Collins, Heather Strachan and Kelley W. Moremen
The Complex Carbohydrate Research Center and the Department of Biochemistry and
Molecular Biology, University of Georgia, Athens, Georgia 30602.

We have cloned, expressed, purified, and characterized a human cDNA encoding a novel α-mannosidase with sequence similarity to members of the CAZY glycosylhydrolases family 38, with greatest sequence and biochemical similarities to the broad specificity human lysosomal α-mannosidase (LysMan). In contrast to prior reports indicating restricted transcript expression patterns for the novel α-mannosidase gene in male mouse and pig reproductive tissues, transcripts encoding the human enzyme were ubiquitously expressed in all human tissues examined. The novel human α-mannosidase studied here, as well as the broad-specificity human LysMan showed catalytic activity towards 4-MU-α-Man in the acidic pH range (optimum pH 4.0 for the novel α-mannosidase) and both were strongly inhibited by swainsonine (Ki=121 nM and 76 nM, respectively) and DIM (Ki=720 nM and 350 nM, respectively). Divalent cation studies indicated that the novel α-mannosidase catalytic activity was stimulated by Co^{+2}, but not other cations, in contrast to the broad specificity LysMan, which was not stimulated by Co^{+2} or any other divalent cation tested. Substrate specificity studies using pyridylaminated high mannose oligosaccharides demonstrated that the novel α-mannosidase cannot hydrolyze $Man_{9\text{-}6}GlcNAc_2$-PA oligosaccharides or $GlcNAcMan_5GlcNAc_2$-PA, but can cleave a single mannose residue from $Man_5GlcNAc_2$-PA. In contrast, the broad-specificity human LysMan can cleave $Man_{9\text{-}5}GlcNAc_2$-PA down to $Man_2GlcNAc_2$-PA, but also would not cleave the $GlcNAcMan_5GlcNAc_2$-PA glycan processing intermediate.

We also performed NMR-based time course studies to examine the hydrolysis of $Man_3GlcNAc$ and $Man_3GlcNAc_2$ oligosaccharides by both the novel α-mannosidase and broad-specificity LysMan. The novel α-mannosidase cleaved only the single α1,6-mannose residue from $Man_3GlcNAc$, but would not cleave the equivalent linkage from the $Man_3GlcNAc_2$ oligosaccharide substrate. In contrast, the broad-specificity LysMan cleaved only the single α1,3-mannose residue from either the $Man_3GlcNAc$ or the $Man_3GlcNAc_2$ oligosaccharide, indicating a clear difference in specificity between these two family 38 α-mannosidases. These substrate specificity data, in combination with the low pH optimum for the novel α-mannosidase, indicate that this cDNA encodes the core-specific lysosomal α1,6-mannosidase catalytic activity that has been previously detected in extracts from human and rat tissues and cell lines (1-4). (Supported by NIH grants GM47533 and RR05351)

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(211) **Conditional Regulation of Cytokine Signaling by N-glycosylation**

Emily A. Partridge[1], Ken Lau[1], Cristina I. Silvescu[2], Judy Pawling[1], Vern N. Reinhold[2] and James W. Dennis[1]
[1] 1Samuel Lunenfeld Research Institute, Mount Sinai Hospital, and University of Toronto, 600 University Ave,
Toronto ON, CANADA, M5G 1X5, [2] Department of Chemistry, University of New Hampshire, Durham, NH, 03824, USA.

The Golgi enzyme β 1,6 N-acetylglucosaminyltransferase V (Mgat5) is up regulated in carcinomas and promotes the substitution of N-glycans with poly N-acetyllactosamine , the preferred ligand for galectin-3 (Gal-3). We recently reported that expression of Mgat5 sensitizes cells to multiple cytokines including EGF, PDGF, bFGF, IGF and TGF-β. Gal-3 cross-links Mgat5-modified N-glycans on EGF and TGF-β receptors at the cell surface, and delays their removal by constitutive endocytosis. Thus, conditional regulation of N-glycan processing drives synchronous modification of cytokine receptors, which optimizes cellular responsiveness to extracellular stimuli. Glucose fuels growth and differentiation as a source of carbon and energy, but glucose also supplies positive feedback to cytokine signaling in a poorly understood manner. We show that biosynthesis of galectin ligands is dependent on glucose flux through the hexosamine pathway to UDP-GlcNAc and its supply to Golgi enzymes, notably N-acetylglucosaminyltransferases V (Mgat5). To determine whether glucose supply directly regulates cytokine signaling by regulating cytokine receptor surface levels, Mgat5+/+ mammary carcinoma cells were conditioned in low glucose media. This reduced cytokine responsiveness, lowered L-PHA reactivity, and reduced the levels of surface EGFR and Tβ R receptors. To determine whether the effects of glucose on the receptors are dependent on the hexosamine pathway, cells were grown in low glucose media supplemented with GlcNAc or GlcN. GlcN or GlcNAc supplements elevated cellular UDP-HexNAc levels 10-12 times compared to their non-supplemented counterparts. GlcNAc supplementation restored L-PHA binding, cytokine signaling and maximal levels of cell surface cytokine receptors, consistent with a requirement for the hexosamine pathway in maintaining cellular responsiveness to cytokines. Remarkably, GlcNAc supplements to Mgat5-/- cells enhanced levels of cell surface cytokine receptors, increased levels of poly N-acetyllactosamine substitutions, and consequently rescued cytokine signaling. As cells approach senescence, surface cytokine receptors decline by loss to endocytosis, similar in this regard to the Mgat5 deficiency. If glucose flux through the hexosamine and N-glycosylation pathways provides positive feedback to anabolic cytokine

receptors in vivo, Mgat5-/- mice could be expected to display some metabolic insufficiency and loss of fitness particularly with age. Indeed, Mgat5-/- mice displayed reduced longevity, with signs of premature aging including osteoporosis, lethargy and weight loss. Furthermore, blood glucose in both fed and fasted Mgat5-/- animals at 3 months age was reduced by 10-15% compared to wild type littermates. Low intracellular concentration of glucose in insulin-resistant muscle promotes slow to fast myofiber conversion in skeletal muscle, and muscle in 3-month-old Mgat5-/- mice displayed an increased proportion of fast to slow twitch myofibers. We speculate that the molecular network of hexosamine, Golgi and cytokine signalling pathways adapts to nutrient availability in adult tissues, and may contribute to ageing by regulating stem cell renewal and metabolism. Indeed, comparison of osteogenic potential in bone marrow cultures from age-matched mice revealed a more severe age-related decline in the Mgat5 mutant mice. Our model suggests that Mgat5 supplies positive feedback to anabolic signaling, and in vivo, mice lacking the enzyme display reduced fitness and survival. 1. J. W. Dennis, S. Laferte, C. Waghorne, M. L. Breitman, R. S. Kerbel, Science 236, 582 (1987) 2. M. Pierce, J. Arango, J.Biol.Chem. 261, 10772 (1986) 3. K. Yamashita, Y. Tachibana, T. Ohkura, A. Kobata, J.Biol.Chem. 260, 3963 (1985) 4. J. Hirabayashi et al., Biochim.Biophys.Acta 1572 232 (2002) 5. E. A. Partridge et al., Science in press, (2004)

(212) **The First Fibronection Type III Repeat of NCAM is Necessary for its Recognition and Polysialylation by ST8Sia II (STX) and ST8Sia IV (PST)**

Shalu S. Mendiratta, Brett E. Close, Kristin M. Geiger and Karen J. Colley
Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, College of Medicine
900 S. Ashland Avenue, Chicago, IL 60607.

The polysialyltransferases ST8Sia IV (PST) and ST8Sia II (STX) are responsible for the biosynthesis of alpha 2, 8-linked polysialic acid on the N-linked, and possibly O-linked, glycans of a small subset of glycoproteins. Polysialic acid has been shown to be an anti-adhesive molecule capable of promoting neurite outgrowth, axon guidance and path finding, and general cell migration. Polysialic acid is expressed at high levels in the embryo and neonate. It is down-regulated in the adult animal, but persists in areas of the brain, such as hippocampus and olfactory bulb where continued cell migration, neurogenesis and synaptic plasticity are necessary. Interestingly is it also re-expressed on the surface of cancer cells where it may act to promote metastasis. The small number of proteins that are modified by polysialic acid suggest that it is added in a protein specific fashion. The neural cell adhesion molecule (NCAM) is the major carrier of polysialic acid in mammals. We predict that an initial protein-protein contact between the polysialyltransferases and NCAM is the basis for this protein specificity. NCAM's extracellular domain consists of five immunoglobin-like (Ig) domains and two fibronectin type III (FN) repeats. We have found that the minimal NCAM protein capable of recognition and polysialylation by the PST and STX consists of fifth Ig domain containing the sites for polysialylation and the adjacent FN repeat (NCAM-4). We predict that the first FN repeat (FN1) of NCAM acts as the recognition site for the enzymes. Interestingly FN repeats are found in at least 2% of all animal proteins, however not all of these proteins are polysialylated. To determine whether other FN repeats are capable of replacing FN1 of NCAM, we have generated two chimeric proteins where we deleted FN1, effectively replacing it with FN2 (DFN1), and where we replaced NCAM's C-terminus (FN1, FN2, transmembrane region and cytoplasmic tail) with FN3, FN4, FN5, the transmembrane region and cytoplasmic tail of L1, another adhesion molecule that is not polysialylated (NCAM-L1). In contrast to full length NCAM, DFN1 and NCAM-L1 exhibited little to no polysialylation when co-expressed with ST8Sia IV (PST) even though they were folded and trafficked efficiently to the cell surface. These results suggest that specific features of the FN1 of NCAM are recognized by the polysialyltransferases. Currently we are evaluating which sequences within FN1 are required for NCAM polysialylation. We have modeled FN1 based on the known structure of FN2. Preliminary results suggest that a negatively charged patch in FN1 is required for the efficient polysialylation of our minimal NCAM protein (NCAM-4). Future studies employing direct binding assays will determine whether replacement of these acidic amino acids disrupts the interaction between NCAM and the polysialyltransferases.

(213) **Mechanism of Uptake and Incorporation of N-glycolylneuraminic Acid into Human Cells**

Sandra Diaz, Muriel Bardor, Dzung H. Nguyen and Ajit Varki
Glycobiology Research and Training Center and Department of Medicine and
Cellular & Molecular Medicine, University of California, San Diego.

N-glycolylneuraminic acid (Neu5Gc) is a widely expressed sialic acid in mammalian tissues. Humans are genetically deficient in the machinery for producing Neu5Gc due to a mutation in the CMP-Neu5Ac hydroxylase (CMAH) gene. We recently reported that free Neu5Gc can be incorporated into cultured human carcinoma cells in vitro, and showed evidence for small amounts of Neu5Gc in normal human epithelial and endothelial cells in vivo. Absent any proven alternate pathway for Neu5Gc synthesis in humans, we suggested that this Neu5Gc originates from dietary sources. We now need to understand the uptake pathway(s) of Neu5Gc and its potential precursor ManNGc, into human cells. Current studies allow us to conclude that the Neu5Gc uptake mechanism is not specific for human carcinoma cells, but also occurs in other cultured human and mammalian cell lines. HPLC and MS experiments prove that the membrane-associated acid-released sialic acid is indeed Neu5Gc. Isolation of high molecular weight components analyzed by HPLC and western blot analysis show that Neu5Gc is incorporated in proteins. Also, inhibitors of certain non-clathrin mediated endocytic pathways reduce Neu5Gc accumulation. Studies with human mutant fibroblasts show that the lysosomal sialic acid transporter is required for the incorporation of free Neu5Gc, but not ManNGc into cells. Competition experiments confirm that Neu5Ac and Neu5Gc are taken up by the same transporter. Metabolic incorporation of glycosidically-bound Neu5Gc from exogenous glycoproteins required the presence of the transporter, as well as the lysosomal sialidase, which is presumably needed to release free Neu5Gc from the glycoproteins in the lysosome. Neu5Gc accumulation in lysosomes is observed in mutant fibroblasts that lack activity of either the lysosomal sialic acid transporter or lysosomal sialidase. Taken together, our data indicate that exogenous Neu5Gc enters cells via multiple endocytic pathways, reaches lysosomes, and is exported in free form into the cytosolic compartment by the lysosomal sialic acid transporter. There, it is available for activation and eventual transfer to glycoconjugates. This mechanism for the uptake of free Neu5Gc into cultured cells can also explain prior reports of efficient incorporation of unnatural sialic acids into cells. In contrast, ManNGc likely traverses the plasma membrane by passive diffusion and becomes available for conversion to Neu5Gc in the cytosol.

(214) **Identification of the Occupied N-glycan sites of β_1 Integrin by ^{18}O Back Labeling**

Avinash H. Sujan, Gerardo A Manilla, Intaek Lee, Dr. Lance Wells and Dr. Micheal Pierce
3059 CCRC, 315 Riverbend Road, Athens, GA 30605.

Many cell surface receptors involved in cell-adhesion, migration and signal transduction events through interactions with the extracellular matrix are heterodimeric transmembrane glycoproteins called integrins. Studies focusing on the roles of integrin subunit N-glycosylation have identified that the N-glycans play a functional role in integrin ligand binding activity as well as the non-covalent interaction between the subunits to form an active cell surface receptor (1). Recent research suggests that there is a positive correlation between the aberrant glycosylation of β_1 integrin N-glycans, found on some transformed cells, with reduced integrin clustering, cell matrix interaction and stimulation of cell migration (2). However, despite recent findings highlighting the functional importance of integrin receptor N-glycans, little information is available with respect to the identity of the occupied N-glycan sites on any of the integrin subunits. Therefore, in our study we will identify the occupied N-glycosylation sites for β_1 integrin, the most widely associated β subunit found in integrin receptors. The glycoprotein after reduction and carboxy-methylation is proteolysed (Lys-C followed by trypsin, in order to reduce chances of missed cleavage by trypsin), desalted and dried. The digested protein is then resuspended in $H_2{}^{18}O$ and treated overnight with PNG-ase F. N-glycanase cleavage of the N-glycans in the presence of ^{18}O converts the glycosylated asparagines (asn) to aspartic acids (asp) (= +1 m.u.) and also incorporates an ^{18}O in the asp carboxylic acid (= +2 m.u.), thereby back-labeling all occupied N-glycan sites with ^{18}O. The three mass unit increase in the molecular weight of the peptide that was N-glycosylated prior to N-glycanase treatment is then identified by LC-Linear Ion Trap mass spectrometry. The identity of the occupied sites will then be used in molecular modeling studies in order to identify the three dimensional position of each site in the glycoprotein and thereby provide some insight into which sites are more susceptible to the presence of aberrant

glycosylation in transformed cells as well as which occupied sites maybe involved in the non-covalent interaction between the subunits forming a functional integrin receptor.

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(215) **Mechanism of Substrate Binding and Catalysis for Class I (glycosylhydrolase family 47) α1,2-mannosidases.**

Khanita Karaveg[1,2], Aloysius Siriwardena[2], Wolfram Tempel[1], Zhi-Jie Liu[1], Bi-Cheng Wang [1] and Kelley W. Moremen[1,2]

[1] Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602, [2] Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602.

Mammalian Class I α1,2-mannosidases (glycosyl hydrolase family 47) play critical roles in the maturation of Asn-linked glycoproteins in the endoplasmic reticulum (ER) and Golgi complex as well as influencing the timing and recognition for disposal of terminally misfolded proteins during ER-associated degradation. Class I mannosidases can be categorized into three subfamilies: 1) ER mannosidase I subfamily hydrolyzes a single mannose from $Man_9GlcNAc_2$ to generate a specific $Man_8GlcNAc_2$ isomer B, 2) Golgi mannosidase I subfamily members hydrolyzes $Man_{8-9}GlcNAc_2$ to $Man_5GlcNAc_2$, which is a substrate for GlcNAc transferase I as the committed steps in the synthesis of complex-type oligosaccharides, and 3) EDEM proteins that do not appear to have an intrinsic hydrolase activity, but appear to play a significant role in the disposal of terminally misfolded glycoproteins in the ER through the presumed recognition of $Man_8GlcNAc_2$ glycan structures. The proposed role of the $Man_8GlcNAc_2$ isomer B processing intermediate in targeting misfolded proteins for ER-associated degradation suggests that both ER mannosidase I and EDEM homologs are potential therapeutic targets for inhibition in the treatment of ER storage disorders. Despite several recent reports of X-ray structures of class I mannosidases, the proposed catalytic mechanism has not yet been experimentally investigated. We have chosen human ER mannosidase I as a candidate enzyme to investigate Class I mannosidase catalysis and binding kinetics. Single and double mutants of three putative catalytic (E330, D463 and E599) and the glycone binding (F569 and T688) residues were investigated for their impacts on catalysis and substrate binding. The kinetics of binding for a D463N mutant to $Man_9GlcNAc_2$ was analyzed by surface plasmon resonance indicating that this residue is mainly responsible for substrate binding, but not catalysis. pH-rate dependence analysis suggested that there are four amino acids responsible for the macroscopic dissociation constants during substrate binding and catalysis. The optimum pH and pK_a shift observed in the E330Q/A mutants strongly indicate that E330 is the general acid catalyst. A proton inventory study gave a KIE of 1.8±0.2, but did not resolve the involvement of a second water residue previously proposed from X-ray structure studies. The presumed general base catalyst is E599 based on the isolation and X-ray structure determination of a co-complex between an α1,2 mannobiose thio-disaccharide substrate analog and human ER mannosidase I resolved to 1.4 Å. The uncleaved thio-disaccharide co-complex bridges the enzyme +1 and -1 subsites and reveals a unique $^3S_1$ sugar ring conformation for the -1 subsite residue. This conformation, in combination with the prior identification of a $^1C_4$ sugar ring conformation for 1-deoxymannojirimycin in the -1 subsite, suggests that the Class I mannosidases employ a novel $^3H_4$ sugar conformation in the -1 subsite at the catalytic transition state. Potential roles for additional residues adjacent to the catalytic carboxyl side chains are also proposed to influence the ionization state during acid/base catalysis. (Supported by NIH grants GM47533 and RR05351)

(216) **Cosmc Prevents Aggregation/Proteasomal Degradation of Mammalian T-synthase**

Tongzhong Ju, Caleb Stowell and Richard D. Cummings

Department of Biochemistry & Molecular Biology and Oklahoma Center for Medical Glycobiology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104 .

Mucin-type O-glycans are synthesized by glycosyltransferases primarily localized in the Golgi apparatus. A key precursor O-glycan is the core 1 structure Galβ1-3GalNAcα1-Ser/Thr (or T-antigen), which is synthesized by the core 1 β3-galactosyltrasferase (Core 1 β3-Gal-T or T-synthase) acting on the Tn antigen GalNAcα1-Ser/Thr. We recently identified a unique molecular chaperone termed Cosmc (Core 1 β3-Gal-T Specific Molecular Chaperone) that is required for the activity of mammalian T-synthase. We have now shown that Cosmc is an ER-localized, ATP-binding protein, but the molecular mechanism(s) through which Cosmc promotes maturation and activity of the T-synthase is unknown. Here we report that Cosmc prevents the aggregation of mammalian T-synthase, leading to maturation and correct dimerization of the active enzyme. LSC cells, a human colon carcinoma cell line, contains a normal T-synthase gene, but lacks T-synthase activity due to an insertional mutation in the Cosmc gene. Thus, LSC cells are unable to generate active T-synthase, and consequently express Tn and sialyl-Tn antigens. Introduction of wt-Cosmc into LSC cells restores the activity of endogenous T-synthase and allows the cells to express the sialyl-T antigen. Recombinant T-synthase expressed in LSC cells is degraded and cleaved (truncated) and is inactive. In contrast, LSB cells, a related colon carcinoma cell line, contain both normal Cosmc and T-synthase genes, and can express a full-length and active form of recombinant T-synthase. Sucrose gradient subcellular fractionation showed that the truncated form of recombinant T-synthase in LSC cells resides in heavy membrane fractions (RER) while recombinant T-synthase expressed in LSB cells is in light membrane fractions (Golgi). LSC cells treated with the proteasomal inhibitors MG-132 or lactacystin accumulate full-length recombinant T-synthase, but the accumulated enzyme remains inactive. In the absence of functional Cosmc, the truncated form of T-synthase expressed in LSC cells is RER-associated. When extracts of LSC and LSB cell expressing recombinant T-synthase were treated with DSS, a noncleavable protein crosslinker, all of the T-synthase expressed in LSC cells was recovered in a cross-linked, high-molecular weight oligomeric complex. By contrast, T-synthase expressed in LSB cells is recovered under these conditions as monomeric/dimeric species. In a similar fashion, we found that recombinant T-synthase expressed in Hi-5 insect cells, which lack endogenous Cosmc, is also inactive and is recoverable in a cross-linked, high-molecular weight oligomeric complex. By contrast, recombinant T-synthase co-expressed with wt-Cosmc in Hi-5 insect cells is recovered as a monomeric/dimeric species. These results demonstrate that Cosmc prevents the RER aggregation and proteasomal degradation of mammalian T-synthase, thereby promoting the proper folding and acquisition of T-synthase activity. Because the Cosmc gene is X-linked (Xq23), its somatic mutation may be related to several human diseases (autoimmune diseases and cancer) that are associated with expression of the Tn antigen.

(217) **Protective Effects of Model Maillard Reaction Products on Metal-ion Induced Cytotoxicity.**

David Kitts.

Food, Nutrition and Health, University of B.C. Vancouver, B.C., V6T 1Z4 Canada.

Two models, one comprised of PM2 bacteriophage DNA and another comprised of C3H10T1/2 mouse embryo fibroblast cells were used to study the potential of nondialysable model Maillard Reaction Products (MRPs; mw>3.5kD), derived from glucose-lysine (Glu-Lys); fructose-lysine (Fru-Lys), and ribose-lysine (Rib-Lys) reactants (0.001-0.1% w/v) to block polyvalent metal (Cu^{2+}, Fe^{2+}, Fe^{3+}@ 0.1-50 μM)- induced genotoxicity and cytotoxicity. All three model MRPs reduced DNA breakage induced by 0.1-10 μM Fe^{2+} and Cu^{2+}, only. Fru-Lys MRPs at both low (0.001%) and high (0.01%) concentrations, significantly ($P<0.05$) enhanced the DNA breaking activity of Fe^{3+}. Different results were observed with the mouse embryo fibroblast culture experiments. All three MRPs at both (e.g. 0.001% and 0.01%, w/v) concentrations enhanced ($P<0.05$) the colonization efficiency (CE) of cultured fibroblast cells in the presence of 0.1 and 10 μM Fe^{2+} and Cu^{2+}, respectively. In contrast, the application of MRPs at 0.001% w/v concentrations with Fe^{3+} (0.1 and 10 μM), lowered ($P<0.05$) the fibroblast CE. The cytotoxic effect of MRPs with $Fe^{3+}$was only lowered at a high concentration of MRPs (0.01%, w/v) following a pre-mixing treatment of Fe^{3+} with MRPs. Model MRPs were shown to selectively protect against polyvalent metal ion-induced genotoxicity and cytotoxicity, presumably by chelation of metal ions. Supported by a NSERC-discovery grant.

(218) **Substrate Specificity of Golgi a-Mannosidase IIx Indicates a Functional Redundancy with Golgi a-Mannosidase II in Animal Tissues**

Harminder Singh[1], Heather Strachan[1], Michiko N. Fukuda[2] and Kelley W.

Moremen[1]
[1] Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602-4712,
[2] The Burnham Institute, La Jolla, California 92037.

Glycoprotein maturation in the Golgi apparatus produces unique carbohydrate structures through the action of a complex array of glycosidase and glycosyltransferase activities that are expressed in a tissue-specific manner. Defects in these pathways can impair normal development leading to anemia, autoimmune disorders, mental retardation, male infertility or death. We have been analyzing the substrate specificities of several Golgi glycosidases in order to establish their roles in N-glycan maturation. Two homologous enzymes, Golgi a-mannosidase II (GMII) and Golgi a-mannosidase IIx (GMIIx), have been hypothesized to play roles in the last mannose trimming steps in the N-glycan maturation pathway. GMII catalyzes the committed step in the conversion of hybrid to complex glycoproteins by converting $GlcNAcMan_5GlcNAc_2$ to $GlcNAcMan_3GlcNAc_2$. Prior data has indirectly indicated that GMIIx catalyzes an alternate processing pathway that is not dependent on the action of GlcNAc transferase I, however, direct demonstration of ManIIx specificity toward high mannose oligosaccharides has not been reported. During our analysis of cDNA clones and EST sequences encoding GMIIx, we noticed a number of apparent splice variants within the coding region. Each of the GMIIx splice variants were expressed in HEK 293 cells as soluble secreted catalytic domains and assayed for a-mannosidase activity using 4-MU-a-Man as a substrate. Expression of one splice variant resulted in the secretion of an active enzyme that was purified from the conditioned culture media using a combination of Phenyl Sepharose, Blue Sepharose, Hydroxyapatite and Superdex 200 column chromatography. The purified recombinant GMIIx catalytic domain was active from pH 5-7, with a pH optimum of 5.6, and was stimulated 2.6-fold by 5 mM Co^{2+}. GMIIx was inactive against the natural pyridylaminated oligosaccharide substrates $Man_9GlcNAc_2$-PA, $Man_6GlcNAc_2$-PA and $Man_5GlcNAc_2$-PA, but was able to cleave $GlcNAcMan_5GlcNAc_2$-PA to $GlcNAcMan_4GlcNAc_2$-PA and $GlcNAcMan_3GlcNAc_2$-PA. These data indicate that GMIIx has an oligosaccharide substrate specificity quite similar to GMII and that these enzymes are likely to be functionally redundant in most mammalian tissues. (Supported by NIH grants GM47533 and RR05351 to K.W.M. and CA71932 to M.N.F.)

(219) **Functional Analysis of Amino Acids Within the Alpha 1,3Fucosyltransferase Motif**

Kyung I. Lee and Bruce A. Macher
Department of Chemistry, San Francisco State Univ., 1600 Holloway Avenue, San Francisco, CA 94132 .

During an inflammatory response, selectins expressed on the surface of endothelial cells bind to ligands expressed on the surface of leukocytes to initiate the process of extravasation. Alpha 1,3fucosyltransferases (FTs) catalyze the final step of the synthesis of these selectin ligands. Alignment of the catalytic domain of FTs found in various species has revealed a conserved region known as the alpha 1,3FucT motif, originally described by Martin and coworkers (Martin et al. 1997, JBC vol. 272). We have previously characterized the function of the amino acids within this motif by alanine scanning. Only four of the Ala mutants had sufficient enzymatic activity for kinetic analysis. Those six mutants with low or no enzyme activities were conservatively mutated to elucidate the role of these amino acids. Each of the conservative mutants was enzymatically active, and acceptor Km for the mutants ranged from 2- to 40-fold higher than that of the wild type FT. Preliminary results indicate that three of the six mutants have a 2- to 6-fold increase for the donor Km compared to the wild type FT.

(220) **GalNAc-transferase T3 (GalNAc-T3) gene regulation in HT29 and ASPC-1 cells.**

Eric P. Bennett[1], Mads A. Tarp[1], Johannes W. Pedersen[1], Tina Skjoerringe[1] and Michael A. Hollingsworth[2]
[1] School of Dentistry, University of Copenhagen, Noerre Alle' 20, 2200N, Denmark, [2] Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, USA.

A large polypeptide GalNAc-transferase gene family controls mucin-type O-glycosylation. GalNAc-transferase isoforms are differentially expressed temporally and spatially in both normal and malignant cells (Kingsley et al.,2000, Mandel et al. 1999). Current understanding of the molecular events controlling expression of GalNAc-transferases is limited to one report describing a minimal promoter element controlling expression of GalNAc-T3 (Nomoto et al. 1999). Regulation of GalNAc-T3 expression is of particular interest since studies have indicated that GalNAc-T3 expression in cancer is associated with improved survival (Onitsuka et al., 2003, Dosaka-Akita et al., 2001, Shibao et al., 2002, Miyahara et al., 2004). Furthermore, GalNAc-T3 expression in pancreatic adenocarcinoma cell lines was inversely correlated with metastatic potential (Sutherlin et al., 1997). In order to shed light on the regulatory elements controlling GalNAc-T3 gene expression we have further studied the GalNAc-T3 promoter and found two additional potential TATA less regulatory elements separated 20Kbp apart. Of these one minimal 555bp promoter element, among others containing multiple SP1 transcription factor binding sites, was found to drive expression of a luciferase construct in both in human colonic HT29 cells and pancreatic ASPC1 cells, however, only HT29 express GalNAc-T3. GalNAc-T3 mRNA and protein were undetectable in ASPC1 cells. This suggests that transcription of GalNAc-T3 in ASPC1 may be regulated by unknown silencer/repressor elements. Further studies are in progress to delineate the molecular basis for expression of GalNAc-T3 in cancer cells.

(221) **Cloning and Functional Analysis of O-mannosyltransferase that Synthesizes O-linked Mannose in *Aspergillus fumigatus***

Cheng Jin[1], Hongyan Hu[1] and Ruoyu Li[2]
[1] State Key Laboratory of Microbial Resources,Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China, [2] Medical Division, Peking University, Beijing 100080, China.

Aspergillus fumigatus is one of the most ubiquitous of the airborne saprophytic fungi, which has been shown to be an opportunistic pathogen causing pneumonia and other fatal invasive infections in immuno-compromised hosts, particularly among patients undergoing cytotoxin chemotherapy or bone-marrow transplantation. There has been a dramatic increase in severe and usually fatal invasive aspergillosis caused by A. fumigatus. However, the glycosylation occurred in A. fumigatus is almost totally unknown.Recenetly, we cloned an O-mannosyltransferase from A. fumigatus, which is responsible for O-mannosylation of secreted proteins. By replacement with pyrG gene, the O-mannosyltransferase gene was knock-out and a mutant was obtained. The mutant exhibited altered spore-differtiation, also the hyphal growth was observed temperature-sensitive. Using mouse model, the ability for infection caused by the mutant was investigated.

(222) **Evidence for the Existence of Two Distinct Oligosaccharyl Transferase Complexes in the Yeast Saccharomyces cerevisiae**

Aixin Yan, Elain Wu and William J. Lennarz
Department of Biochemistry and Cell Biology and the Institute for Cell and Developmental Biology, State University of New York at Stony Brook, New York.

In eukaryotic cells membrane bound and secretory glycoproteins are initially synthesized on ribosomes, recognized by the signal recognition particle (SRP) and directed to the endoplasmic reticulum (ER) via the translocon machinery. During elongation and translocation, the nascent protein is recognized by the oligosaccharyl transferase complex (OT) and is N-glycosylated at the selected consensus sequence of -Asn-X-Thr/Ser-. It is known that there are three structurally similar translocons in the ER membrane: the trimeric Sec61p, as well as the trimeric Ssh1p complex, which both function in co-translational protein translocation and the heptameric Sec62/63 translocon complex which functions in post-translational process. We asked if there is more than one OT complex in the ER membrane that might specifically associate with these structurally similar but functionally different translocons. We utilized the split-ubiquitin system, a powerful two-hybrid approach to determine membrane protein interactions, to explore the possible oligomerization of OT subunits in yeast. The system uses the two halves of ubiquitin, the N-terminus of ubiquitin (Nub) and C-terminus of ubiquitin (Cub) fused to a reporter gene (R). Wild type Nub spontaneously assembles with Cub-R resulting in proteolytic cleavage and subsequent activation of the reporter gene, whereas mutant Nub (mNub) is not able to assemble with Cub-R unless other proteins that interact with each other are fused to the Cub-R and the mNub. Using this technique, we first established the membrane topology of all of 9 yeast subunits. Next, we fused mNub and Cub-R respectively to two copies of the same protein to determine if it dimerizes or oligomerizes. It was found that except for Ost3p and Ost6p, all the other OT subunits were shown to exist at least as dimers. Moreover, Ost3p and Ost6p display the

same interaction pattern with all the other OT subunits detected both by the split-ubiquitin assay and by cross-linking experiments. Further studies revealed that Ost3p and Ost6p do not interact with each other. It was known that co-immunoprecipitation of the cell lysate of a strain where Ost3p is HA tagged results in precipitation of all other OT subunits except Ost6p. We carried out the same experiments on Ost6p using Ost6p HA tagged strain and the converse results were observed. Furthermore, a previous genetic study demonstrated that disruption of OST3 or OST6 causes only a minor defect in N-glycosylation whereas a ¶§ost3¶§ost6 double mutant displays a very severe under-glycosylation of soluble and membrane-bound glycoproteins. All these results together provide strong evidence that there are two forms of the OT complex in the ER membrane, differing in the presence of either Ost3p or Ost6p. It has been demonstrated that the three translocons in the ER membrane differ with respect to the hydrophobicity of the signal sequences they recognize. We speculate that OT, as one of the most important components of the protein modification machinery, exists as an oligomer and might associate with specific forms of translocons to facilitate glycoprotein synthesis in the ER. (Supported by NIH Grant GM 33185 to WJL)

(223) **Components of the Soldier Defense Gland of the Formosan Subterranean Termite: Coptotermese formosanus: Unusual Occurance of a new 5000 Da Lysozyme with No Homology to Known Lysozymes**

Youshong Guo[1], Masaya Ohta[2], Marcus Hardt[1], Gregg Henderson[3] and Roger A Laine[1,3]

[1] Division of Biochem. and Mol. Biol., Dept. of Biological Sciences, Louisiana State University and the LSU Agricultural Center, Baton Rouge, LA 70803, [2] Department of Biochemistry and Pharmacology, Fukuyama University, Fukuyama, Japan, [3] Department of Entomology, Louisiana State University Agricultural Center, Baton Rouge, LA 70803.

The Formosan Subterranean Termite was imported to the Southern USA in the 1940's and 1950's. Having no natural predators, and with native trees having little resistance, this organism has spread throughout Louisiana and surrounding states. As many as 30% of the Live Oak trees in some areas of New Orleans may be infested, and the destruction to forests, wooden structures and buildings is estimated in $Billions annually. The soldiers comprise 20-25% of the caste system, and are helpless to feed, having mandibles modified for defense. Thus, 25% of the population must be fed by the worker caste. The frontal defense gland makes up 1/3 of the body mass of the soldier and begins as a cavity in the head extending back through the body. An opening in the front of the head is used for discharging the contents of the defense fluid on attacking predators such as ants. Obviously the defense secretion alone makes up more than 7% of the biomass of the nest, a significant metabolic and genetic investment. Nothing is known about the function of any component of the defense secretion. We began to study the contents of the secretion recently, and have found several classes of small molecules and as many as 9 new proteins. The small molecules interestingly include free ceramide with a variety of long chain bases and free fatty acids. The secretion repels workers, attracts soldiers and stimulates transformation of workers to soldiers when they have subsequent molts after exposure to the defense fluid. It is interesting that a defense reaction which may result in the loss of soldiers, stimulates production of new soldiers. The proteins may form a kind of immobilization glue that impedes and irritates invaders. We have found a novel lysozyme that appears to have no homology to known lysozymes or other proteins in the drosophila genome. This lysozyme may act as an antibiotic in the large tank of defense fluid. We have also discovered 2 putative lipocalins in the secretion, which may function to carry lipid components, pheromones or other, as yet, unidentified molecules. This work has been supported by the USDA-ARS.

(224) **Unique Catabolic Pathway of Glycosphingolipids in a Hydrozoan, *Hydra magnipapillata*, Involving Endoglycoceramidase**

Yasuhiro Horibata[1,6], Keishi Sakaguchi[1], Nozomu Okino[1], Hiroshi Iida[2], Masanori Inagaki[3], Toshitaka Fujisawa[4], Yoichiro Hama[5] and Makoto Ito[1]

[1] Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, [2] Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, [3] Faculty of Pharmaceutical Sciences, Graduate School Kyushu University, [4] Department of Developmental Genetics, National Institute of Genetics, [5] Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, [6] The present address is the Neuronal Circuit Mechanism Research Group, RIKEN Brain Science Institute.

Endoglycoceramidase (EGCase; EC 3.2.1.123) is an enzyme capable of cleaving the glycosidic linkage between oligosaccharides and ceramides of various glycosphingolipids. We detected strong EGCase activity in animals belonging to *Cnidaria, Mollusca* and *Annelida*, and cloned the enzyme from a hydra, *Hydra magnipapillata*. The hydra EGCase, consisting of 517 amino acid residues, showed 19.2% and 50.2% identity to the *Rhodococcus* and jellyfish EGCases, respectively. The recombinant hydra enzyme, expressed in CHOP cells, hydrolyzed [^{14}C]GM1a to produce [^{14}C]ceramide with a pH optimum at 3.0-3.5. Whole mount *in situ* hybridization and immunocytochemical analysis revealed that EGCase was widely expressed in the endodermal layer, especially in digestive cells. GM1a injected into the gastric cavity was incorporated and then directly catabolized by EGCase to produce GM1a-oligosaccharide and ceramide, which were further degraded by exoglycosidases and ceramidase, respectively. However, hydra exoglycosidases did not hydrolyze GM1a directly. These results indicate that the EGCase is indispensable for the catabolic processing of dietary glycosphingolipids in hydra, demonstrating the unique catabolic pathway for GSLs in the animal.

(225) **Production of Glycolipid Glucosylceramide by the Yeast *Saccharomyces kluyveri***

Naoya Takakuwa[1], Masahiko Tamura[2], Osamu Matsumoto[2], Masao Ohnishi[3] and Yuji Oda[1]

[1] Department of Upland Agriculture, National Agricultural Research Center for Hokkaido Region, Memuro, Kasai, Hokkaido 082-0071, Japan, [2] Shimidzu Yeast Manufacturing Plants, Nippon Beet Sugar Manufacturing Co., Shimidzu, Kamikawa, Hokkaido 089-0103, Japan, [3] Department of Agriculture and Life Science, Obihiro University of Agricultural and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan.

Background and Objective: Commercially available preparations of ceramide used as a constituent in cosmetics or food supplements are extracted from plants and principally composed of glycolipid glucosylceramide. The content of glucosylceramide is so limited (0.01-0.09% as dry basis) that the costs for extraction and purification elevate the retail price of a final product by as much as $2-4 per g, even when a product contains as little as 3% glucosylceramide. Glucosylceramide is also found in eukaryotic microorganisms and expected to be produced by using yeast cells. However, the species containing glucosylceramide were putative pathogenic yeasts, such as *Candida* and *Cryptococcus*, which were not the strains used for brewing, baking, or other food processing. *Saccharomyces cerevisiae* synthesizes derivatives of inositol-phosphoryl ceramide instead of glucosylceramide. We have previously surveyed the genera *Saccharomyces*, *Torulaspora*, *Zygosaccharomyces*, and *Kluyveromyces* and found glucosylceramides in *S. kluyveri*, *Z. cidri*, *Z. fermentati*, *K. lactis*, *K. thermotolerans*, and *K. waltii* [Takakuwa *et al*. *FEMS Yeast Res*. 2 (2002) 533-538.]. These observations suggested that certain yeasts could be used for efficient microbial production of glucosylceramide. In the present experiments, we have selected a strain of *S. kluyveri* to produce glucosylceramide efficiently and studied its growth conditions on molasses as the sole carbon source, such as in propagation of commercial baker's yeast. Methods: Twenty-two strains classified as *S. kluyveri* were obtained from NBRC (Japan) and CBS (Netherlands). Yeast cells were grown in the medium containing 5.0% sugar as beet molasses, 0.3% ammonium sulfate, 0.19% urea, 0.075% KH_2PO4, 0.075% $MgSO_4$, and 1.0% corn steep liquor at 30ÅãC for 24 h with shaking (180 rpm). Alkali-stable lipids extracted from the lyophilized cells were separated on TLC developed by chroroform:methanl:acetic acid:water (20:3.5:2.3:0.7, v/v). Glucosylceramide was visualized by orcinol-sulfuric acid reagent, and determined the quantity with Lane & Spot Analyzer (Atto Co., Tokyo). Results: Six strains accumulated relatively high amounts of glucosylceramide (>0.40 mg/g cells as dry basis) and grew well in an Erlenmeyer flask. From strain CBS 4800, 30 strains were isolated from single spores after sporulation and further tested for glucosylceramide contents. Strain SP-25 accumulated higher than the parental strain (0.59 mg/g cells as dry basis) and was finally selected and cultured in a 5-l jar fermentor. In a comparison with a conventional baking strain of *S. cerevisiae*, the selected strain SP-25 propagated more rapidly in conjunction with depressed synthesis of ethanol. Glucosylceramide produced from 1 kg of sugar in molasses was estimated to be 0.15 g. The constituents of glucosylceramide accumulated in the yeast cells were principally 2-hydroxystearic acid as a fatty acid, 9-methyl-*trans*-4, *trans*-8-sphingadienine as a sphingoid base, and glucose as a hexose.

(226) **Regulation of Dpp Signaling by Dally Co-receptor During *Drosophila* Wing Development**

Takuya Akiyama[1], Satomi Takeo[2], Cyndy Firkus[1] and Hiroshi Nakato[1]
[1] Department of Genetics, Cell Biology and Development, The University of Minnesota, Minneapolis, MN 55455, USA, [2] Department of Biology, Tokyo Metropolitan University, Hachioji-shi, Tokyo 192-0397, Japan.

Morphogens are signaling molecules that can specify different cell fates in a concentration dependent manner and play key roles in tissue assembly during development of multicellular organisms. Decapentaplegic (Dpp), a *Drosophila* homologue of bone morphogenetic proteins, has been shown to act as a morphogen during wing development. Several lines of evidence have shown that glypicans, a conserved family of heparan sulfate proteoglycans (HSPGs) attached to the cell membrane via a GPI-linkage, are involved in morphogen gradient formation. We previously demonstrated that Dally, one of the *Drosophila* glypicans, affects the shape of the Dpp 'ligand gradient' (protein distribution) as well as its 'activity gradient' (spatial patterns of signaling activity) in the developing wing. However, the molecular mechanisms by which *dally* regulates the Dpp gradient formation are not well understood. Using the developing wing as our model, we are further studying the molecular basis for spatial control of Dpp signaling by Dally. Here we show (1) Dally colocalizes with Dpp in vivo, (2) Dally and Dpp expressed in *Drosophila* tissue culture cells are coimmunoprecipitated, suggesting that Dally and Dpp form a complex, and (3) Dally enhances Dpp signaling in a cell autonomous fashion. These findings show that Dally meets the criteria for a Dpp co-receptor. We also studied the relationship of receptor and co-receptor in the signaling complex. Dally and Thickveins (Tkv), a type I receptor for Dpp, share common properties as components of the Dpp signaling complex; they both autonomously enhances Dpp signaling and limit migration of Dpp by binding to Dpp protein. Furthermore, co-immunoprecipitation experiments showed that Dally binds to Tkv in vitro, suggesting that these two molecules act together in a signaling complex. Interestingly, *tkv* and *dally* have different effects on the Dpp gradient formation; *tkv* mutations extend the gradient, while *dally* mutations shrink it. Indeed, abnormal Dpp activity gradient in *dally* mutant wing discs was suppressed by heterozogosity of a *tkv* mutation. This indicates that the relative levels of Dally and Tkv are more important than total amount of these molecules to maintain the normal shape of the Dpp gradient. In addition, *tkv* mutations rescued the defective wing vein V of *dally* mutant adults. Together, these results suggest that Dally has an opposite function of Tkv. Thus, Dally has positive and negative effects on the Dpp signal transduction in a context dependent manner.

(227) **Substrate Specificities of Three Distinct Chondroitin/dermatan 4-*O*-sulfotransferases in Human and Zebrafish**

Shuji Mizumoto[1], Naoki Kobayashi[1], Tadahisa Mikami[1], Naohide Kago[1], Hiroshi Kitagawa[1],
Ayumi Miyake[2], Nobuyuki Itoh[2] and Kazuyuki Sugahara[1]
[1] Department of Biochemistry, Kobe Pharmaceutical University, Japan, [2] Department of Genetic Biochemistry, Kyoto University Graduate School of Pharmaceutical Sciences, Japan.

4-*O*-Sulfation of *N*-acetylgalactosamine (GalNAc) residues, which is catalyzed by four chondroitin/dermatan GalNAc 4-*O*-sulfotransferases (C4ST/D4STs), is a high frequency modification of chondroitin sulfate/dermatan sulfate (CS/DS), and was once postulated to be a prerequisite for the conversion of glucuronic acid (GlcUA) into iduronic acid (IdoUA) by C5-epimerase. To investigate the regulatory mechanism of DS biosynthesis, we examined detailed acceptor specificities toward the recognition sugar sequences of human C4ST-1, -2 and D4ST-1 in partially desulfated DS. The soluble recombinant enzymes exhibited differential preferences for the GalNAc residues flanked by either IdoUA or GlcUA in partially desulfated DS polymers. D4ST-1 predominantly utilized GalNAc residues not only in the -IdoUA-GalNAc-IdoUA- but also in the -IdoUA-GalNAc-GlcUA- sequence, whereas C4ST-1 efficiently worked on the GalNAc residues in the -GlcUA-GalNAc-GlcUA-. C4ST-2 could act on the GalNAc residues in both -IdoUA-GalNAc-IdoUA- and -GlcUA-GalNAc-GlcUA-. Therefore, C4ST-2 may possess both properties of C4ST-1 and D4ST-1, suggesting that three human sulfotransferases play different roles in the 4-*O*-sulfation of GalNAc residues in DS biosynthesis. D4ST-1 appears to be essential for the 4-*O*-sulfation of GalNAc residues in IdoUA-rich clusters typical of mature DS chains among three isoforms (Mikami et al., *J. Biol. Chem.* 278, 36115-36127, 2003). In addition, we cloned and characterized their respective zebrafish counterparts of human C4ST/D4STs. Gene knockdown of these genes in zebrafish will reveal the *in vivo* functions of C4ST/D4STs.

(228) **Chondroitin Polymerizing Factor, Which Shows Cell- and Organ-specific Expression in *Caenorhabditis elegans*, is Indispensable for Chondroitin Biosynthesis and Embryonic Cell Division**

Tomomi Izumikawa[1], Hiroshi Kitagawa[1,3], Souhei Mizuguchi[2,3], Kazuko H. Nomura[2,3], Kazuya Nomura[2,3], Jun-ichi Tamura[4], Keiko Gengyo-Ando[3,5], Shouhei Mitani[5] and Kazuyuki Sugahara[1]
[1] Department of Biochemistry, Kobe Pharmaceutical University, Japan, [2] Department of Biological Sciences, Faculty of Science, Kyushu University Graduate School, Japan, [3] Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Agency (JST), [4] Department of Regional Environment, Faculty of Regional Sciences, Tottori University, Japan, [5] Department of Physiology, Tokyo Woman's Medical University School of Medicine, Japan.

Chondroitin polymerization was first demonstrated *in vitro* when human chondroitin synthase (ChSy) was coexpressed with human chondroitin polymerizing factor (ChPF), which is homologous to ChSy but has little glycosyltransferase activity (1). To analyze the biological function of chondroitin, the *C. elegans* ortholog of human *ChSy (sqv-5)* was recently cloned and the expression of its product was depleted by RNA-mediated interferance (RNAi) and deletion mutagenesis (2). Blocking of chondroitin synthesis resulted in defects of cytokinesis in early embryogenesis and eventually cell division stopped. In this study, we cloned the ortholog of human *ChPF* in *C. elegans*, *PAR2.4*, predicting that the mechanism of chondroitin polymerization in *C. elegans* is similar to that in humans. Despite little glycosyltransferase activity of the gene product, chondroitin polymerization was demonstrated as in the case of mammals when PAR2.4 was coexpressed with cChSy *in vitro*. Moreover, the worm phenotypes including the reversion of cytokinesis, observed after the depletion of PAR2.4 by RNAi, were very similar to the *cChSy (sqv-5)*-RNAi phenotypes. Thus, PAR2.4 in addition to cChSy is indispensable for the biosynthesis of chondroitin in *C. elegans*, and the two co-operate to synthesize chondroitin in vivo. The expression of the PAR2.4 protein was observed in seam cells, which can act as neural stem cells in early embryonic lineages. The expression was also detected in vulva and distal tip cells of the growing gonad arms from L3 through to the young adult stage. These findings are consistent with the notion that chondroitin is involved in the organogenesis of the vulva and maturation of the gonad, and also indicative of an involvement in distal tip cell migration and neural development. (1) Kitagawa, H., *et al.* (2003) *J. Biol. Chem.* 278, 23666-23671 (2) Mizuguchi, S., *et al.* (2003) *Nature 423*, 443-444

(229) **Oversulfated Dermatan Sulfate Exhibits Neurite Outgrowth-promoting Activity Toward Embryonic Mouse Hippocampal Neurons**

Tadahisa Mikami[1], Megumi Hikino[1], Andreas Faissner[2], Ana-Cristina E. S. Vilela-Silva[3], Mouro S. G. Pavão[3] and Kazuyuki Sugahara[1]
[1] Department of Biochemistry, Kobe Pharmaceutical University, Japan, [2] Department of Cell Morphology and Molecular Neurobiology, Ruhr-University, Germany, [3] Department of Biochemistry, Federal University of Rio de Janeiro, Brazil.

We have shown the neurite outgrowth-promoting activities of chondroitin sulfate (CS) moiety of DSD-1-proteoglycan (PG) (phosphacan) and oversulfated CS chains, such as shark cartilage CS-D and squid cartilage CS-E, toward embryonic hippocampal neurons (J. Biol. Chem. 273, 3296, 1998). We have proposed that the neuritogenic property of the DSD-1 epitope may be attributable to a distinct CS structure characterized by the disulfated disaccharide D unit, GlcA(2S)-GalNAc(6S). In this study, we assessed neuritogenic potencies of oversulfated dermatan sulfate (DS) preparations purified from hagfish notochord, the bodies of two kinds of ascidians and embryonic sea urchin, which are characterized by the predominant disulfated disaccharide units of IdoA-GalNAc(4S,6S), IdoA(2S)-GalNAc(4S) plus IdoA(2S)-GalNAc(6S), IdoA(2S)-GalNAc(6S), and IdoA-GalNAc(4S,6S), respectively. They exerted marked neurite outgrowth-promoting activities, resulting in distinct morphological features depending on the individual structural features. Such activities were not observed for a less sulfated DS preparation derived from porcine skin, which contains a monosulfated disaccharide unit IdoA-GalNAc(4S) as a predominant unit. The neurite outgrowth-promoting activities of these oversulfated DS preparations and DSD-1-PG were eliminated by the

specific enzymatic cleavage of GalNAc-IdoA linkages characteristic of DS using chondroitinase B. In addition, chemical analysis of the glycosaminoglycan side chains of DSD-1-PG revealed the DS-type structures. These observations suggest that oversulfated CS/DS structures exemplified by the DSD-1 epitope may reflect the physiological neurogenesis during mammalian brain development (J. Biol. Chem. 278, 43744, 2003).

(230) **A Hypothesis on the Evolutionary Relationship Between Vertebrate and Invertebrate β-1,4-galactosyl- and N-acetylgalactosaminyl-Transferases**

Boopathy Ramakrishnan[1,2] and Pradman K. Qasba[1]

[1] Structural Glycobiology Section, LECB, CCR, NCI-Frederick, Frederick, MD 21702, USA, [2] BRP, SAIC-Frederick, Inc.,.

β-1,4-galactosyltransferase-I (β4Gal-T1), is mainly involved in the synthesis of the N-glycans, where in the presence of Mn^{2+} it transfers galactose from the sugar donor UDP-α-D-galactose (UDP-Gal) to the acceptor N-acetylglucosamine, GlcNAc, present at the non-reducing end of the growing N-glycan. However, it exhibits a very poor N-acetylgalactosaminyltransferase (GalNAc-T) activity, where it transfers GalNAc from UDP-GalNAc to GlcNAc. Although β4Gal-T1 is found in all vertebrates, in mammals, during lactation together with α-lactalbumin it is involved in the synthesis of lactose, the 'milk sugar'. In human it exists as a family of seven enzymes (-T1 to -T7), with high sequence similarity among their catalytic domains. Although, all these family members transfer Gal from UDP-Gal to a different acceptor substrates, they exhibit very poor or no GalNAc-T activity. Recently β4Gal-T1 homolog gene has been discovered in many invertebrates such as worms, fly etc. Although these enzymes also have high sequence similarity with the human β4Gal-T1, they exhibit broad donor sugar specificity particularly high GalNAc-T activity. Earlier we have shown that the Tyr289 residue in bovine β4Gal-T1 is responsible for the donor sugar (Gal) specificity and when it is mutated to either Leu or Ile, the mutant exhibits broad donor sugar specificity, importantly high GalNAc-T activity compared to the wild-type. In all the vertebrate (frog, fish, birds, mammals etc.) β4Gal-T genes discovered so far contain Tyr or Phe residue at this position. On the other hand, examination of all the invertebrate homolog of β4Gal-T1 to T6 show that corresponding Tyr289 residue in these genes has been substituted with either Leu or Ile, except for the β4Gal-T7 homolog where this residue remains unchanged as either Tyr or Phe residue. Thus, the presence of the Ile/Leu may be responsible for the high GalNAc-T activity. Interestingly, it has been observed that in invertebrates the N-glycan structures have reduced Gal moiety, and also they have very little glycolipids based on the lactosylceramide structure. Based on these observations we hypothesize that during the evolution, nearly 500 million years ago, the emergence of vertebrate from invertebrate may be associated with the mutation of this Ile/Leu residue to Tyr, thus converting the enzyme into a specific galactosyltransferase with very poor GalNAc-T activity. Such conversion seems to have played an important role in further evolution of the vertebrates, since the addition of Gal moiety of the N-glycan has recruited many new sialyltransferases to complete the N-glycan synthesis and correspondingly many lectins to recognize such N-glycans. Similarly, the glycolipids based on the lactosylceramide are abundant in vertebrate and often involved in several cellular processes. To test this hypothesis we have undertaken the enzymology studies on the wild-type N-acetylgalactosaminyltransferase-I from *Drosophila melanogaster* and its mutant Ile289Tyr. The details will be presented. Funded under Contract No. NO1-CO-12400.

(231) **Structure of the Polysialic Acid Specific endosialidaseNF in Complex with Sialic Acid**

Katharina Stummeyer[1], Achim Dickmanns[2], Martina M¸hlenhoff[1], Rita Gerardy-Schahn[1] and Ralf Ficner[2]

[1] Department of Cellular Chemistry, Medizinische Hochschule Hannover, Hanover, Germany , [2] Department of Molecular and Structural Biology, Georg-August-Universit‰t G¨ttingen, G¨ttingen, Germany.

Polysialic acid -α2,8-N-acetylneuraminic acid) is a dynamically regulated (polySia; poly posttranslational modification of the neural cell adhesion molecule (NCAM) implicated in biological processes like neural development, regeneration, and synaptic plasticity. PolySia is also an oncodevelopmental marker expressed by neuroectodermal human tumours with high malignant potential proposed to support tumour growth and metastasis. Similarly neuroinvasive bacteria causing sepsis and meningitis escape the human immune response by wearing polySia capsules. Protecting the pathogens in the human host, the capsules are simultaneously attachment sites for lytic bacteriophages that are equipped with polySia degrading tailspike proteins. These phage-associated endosialidases are the only known enzymes that specifically degrade polySia and are therefore most important tools in polySia-research. Moreover, recent studies in animals demonstrated, that treatment with endosialidases reduces the metastatic potential of polySia encapsulated tumour cells (1) and prevents bacteraemia and death in mice with systemic E. coli K1 infections (2). We have solved the crystal structure of the endosialidase of bacteriophage K1F (amino acids 246-911) as apoenzyme to 1.9 ≈ resolution as well as in complex with oligomeric sialic acid. The enzyme assembles into an SDS-resistant homotrimer (3) with mushroom like outline that is stabilized by a triple intertwined stalk domain. The unique specificity for polySia evolved from functional combination of three distinct domains: a β-barrel domain, a 6-bladed β-propeller typical for exosialidases and a triple β-helix characteristic for bacteriophage tailspike proteins. Sialic acid binding sites could be identified in the β-barrel domain and the triple intertwined stalk domain. The enzyme appears to be a catalytic trimer that simultaneously binds three polySia chains whereby each chain interacts with all three subunits of the homotrimer. The active site clearly differs from what is found in exosialidases since highly conserved active site residues are not preserved in endoNF. Interestingly, this includes an aspartic acid and a tyrosine residue, which are believed to be key residues of the exosialidase reaction. Based on the active site features and the mode of polySia binding, a substrate assisted catalysis mechanism will be discussed as molecular basis for the high polySia specificity of endosialidases. Moreover, a panel of mutant enzymes generated by site-directed mutagenesis were analyzed and will be presented.

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(232) **Leishmania Major Udp-Galactopyranose Mutase: Characterisation and Validation of a Potential Drug Target**

Barbara Kleczka[1], Anne-Christin Lamerz[1], Hans Bakker[1], Martin Wiese[2], Rita Gerardy-Schahn[1] and FranÁoise H. Routier[1]

[1] Medizinische Hochschule Hannover, Abteilung Zellul‰re Chemie, OE4330, Carl- Neuberg Strasse 1, 30625 Hannover, Germany, [2] Bernhard-Nocht-Institut f¸r Tropenmedizin, Bernhard-Nocht-Strasse 74, 20359 Hamburg, Germany.

Galactofuranose (Galf) is a central component on the cell surface of many human pathogens. It is found in a variety of bacteria (eg. Escherichia coli, Klebsiella pneumoniae, Mycobacteria ssp.), fungi (eg. Aspergillus species) or protozoan parasites (eg. Leishmania species, Trypanosoma cruzi). Importantly, Galf is essential for the life of Mycobacteria and is absent in human, so the enzymes involved in its metabolism are attractive targets for the development of new drugs. In recent years, different enzymes involved in Galf metabolism have been characterised in bacteria. In contrast, the galactofuranosyltransferases involved in Leishmania lipophosphoglycan (LPG) biosynthesis are the only eukaryotic enzymes identified. Moreover, the presence of galactofuranose in the cell surface LPG has been shown to be essential for pathogenicity of Leishmania major. UDP-galactofuranose (UDP-Galf) is the donor of Galf and is synthesised by the UDP-galactopyranose mutase (UDP-Galp mutase). This flavoprotein catalyzes the interconversion of UDP-galactopyranose (UDP-Galp) into UDP-Galf. It would therefore be an ideal drug target. We lately identified and characterised this enzyme in Leishmania major using an in vivo complementation system and an in vitro assay (unpublished data). We have now generated a mutant by targeted gene replacement of both UDP-galactopyranose mutase alleles. Recent reports indicate that elements of the 3'- and 5'- untranslated regions regulate gene expression in Leishmania species. Therefore we replaced precisely the UDP-galactopyranose mutase gene alleles by antibiotic resistant genes. Molecular constructs necessary for this Knock out strategy were created by a combination of PCR and homologous recombination. We are currently characterizing the glycoconjugates of the mutant obtained. Additionally experimental infection of mice and macrophages are performed.

(233) **Immunological Activity of Plant *N*-glycans and Synthesis of Neoglycopeptides and Neoglycolipids Carrying Antigenic Glycans**

Mayumi Sakaguchi[1], Megumi Maeda[1], Fumiko Matsuda[2], Mariko Kimura[3], Mitsuhiro Okano[4] and Yoshinobu Kimura[1,2]

[1] The Graduate School of Natural Science and Technology, Okayama University, [2] Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University, [3] Department of Food System, Kurashiki Sakuyo University, [4] The Graduate School Medicine and Dentistry, Okayama University.

It has been revealed that many plant allergens such as pollen allergens are *N*-glycosylated and the *N*-glycans linked to such glycoallergens carry β-1,2-xylosyl and/or α-1,3-fucosyl residues. We have already determined the structures of such antigenic *N*-glycans linked to some allergenic pollen glycoproteins. However, it is still obscure that the antigenic *N*-glycan itself can be a relevant epitope for IgE or whether such *N*-glycans have physiological activity involved in the human cellular immunity. In this study, therefore, we tried to find new immunological function(s) of the plant *N*-glycans. Furthermore, we have developed a synthesis method of neoglycopeptides and glycolipids bearing the antigenic glycans. Inhinition ELISA using plant antigenic *N*-glycans showed that the binding of IgE to a pollen allergen, Cry j1, could not be significantly inhibited by an addition of some antigenic free *N*-glycans, suggesting that the antigenic glycan itself can not be an effective epitope for IgE. This results seems to suggest that certain conformation constructed by the peptide moiety near the glycosylation site and the *N*-glycans would be necessary for the IgE binding. On the other hand, we have found that the antigenic plant *N*-glycan, Man3Xyl1Fuc1GlcNAc2 (M3FX), could suppress a production of IL-4 from T-cells of pollinosis patients, which were previously stimulated by the pollen allergen. This observation seems to indicate that the antigenic *N*-glycan might be a candidate of glyco-drugs for a pollinosis therapy.

For the synthesis of glycolipids bearing antigenic glycans, the sugar chains were acetylated and coupled with 1-octanol using BF_3 as a catalyst in CH_2Cl_2. The acetylated noeglycolipids were finally deacetylated. On the other hand, for the synthesis of glycopeptides, M3FX was conjugated with polylysine by reductive amination. The resulting neoglycopeptides bearing a few of M3FX residues was purified by RP-HPLC. We will discuss the immunological activity of the neoglycolipid or neoglycopeptides. [Reference] Okano, M., Kimura, M., *et al., Clin., Exp., Allergy.* 34, 770-778 (2004)

(234) **The Endogenous Assembly Domain of Endosialidases is Also Present in Tail Spike Proteins of Diverse Bacteriophages**

David Schwarzer, Katharina Stummeyer, Rita Gerardy-Schahn and Martina Muehlenhoff

Abteilung Zellul‰re Chemie, Zentrum Biochemie, Medizinische Hochschule Hannover, Carl-Neuberg-Strafle 1, 30625 Hannover, Germany.

Endosialidases, the only known enzymes that specifically degrade polysialic acid, are tail spike proteins of bacteriophages infecting *Escherichia coli* K1. Endosialidases play a central role in the infection cycle of K1-phages, being involved in phage attachment, penetration of the polysialic acid capsule, and anchoring the phage particle to the outer membrane of the host. Comparison of the primary sequences of different endosialidases demonstrated a modular composition of these proteins. The conserved catalytic domain is flanked by a variable N-terminal and a short C-terminal part that is released by proteolytic cleavage at a highly conserved serine residue. Presence of the intact C terminal domain in the nascent protein is essential for assembling the active trimer but is dispensable in the mature enzyme [1]. However, proteolytic cleavage of the C-terminal domain is not a prerequisite for activity and using purified enzymes we found even an increased molar activity for the non-cleavable mutant Ser911Ala compared to the wild-type. To further characterize the C-terminal part, we performed circular dichroism studies using the separately expressed C-terminal domain of endoNF and the domain isolated after proteolytic processing. Identical spectra were obtained for both, demonstrating that the C-terminal part folds as an independent domain. In contrast to the central part of endoNF which is composed exclusively of β-fold structures [2], the C-terminal domain was found predominantly α-helical. Interestingly, the C-terminal domain shows sequence similarities to the C-terminal domain of tail spike proteins of phages with different host specificities, including the L-shaped tail fibre protein (LTF) of Coliphage T5, the neck appendage precursor protein (gp12) of Bacillusphage GA-1, and the K5 eliminase of an *E. coli* K5 strain. We demonstrated that all three proteins are also cleaved behind the highly conserved serine residue and an exchange to alanine resulted in non-cleavable proteins. In summary, we provide clear evidence that the C-terminal domain is a general assembly domain fused to several tail spike proteins by horizontal transfer of the corresponding gene fragment.

(235) **N-glycans and endo-β-N-acetylglucosaminidase in Rice Cultured Cells**

Megumi Maeda[1] and Yoshinobu Kimura[1,2]

[1] The Graduate School of Natural Science and Technology, Okayama University,

[2] Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University.

Free *N*-glycans have been found in various plant cells, especially in developing or growing cells. As a part of study to reveal the physiological function(s) of such free *N*-glycans and the de-*N*-glycosylation mechanism in plant cells, we have purified and characterized endo-β-*N*-acetylglucosaminidase (endo-OS) from rice cultured cells. Furthermore, we analyzed the structures of unconjugated and conjugated *N*-glycans occurring in rice cultured cells.

Endo-OS having a molecular weight of about 70 kDa showed a strong activity toward the high mannose-type *N*-glycans with the Manα1-2Manα1-3Manβ1-unit as well as other plant endoglycosidases. This result suggests plant endoglycosidases have a common specific subsite to recognize the structural unit, which enhances the reaction rate. Furthermore, we have determined the amino acid sequences of some peptides purified from a trypsin digest of the endo-OS. Some of the amino acid sequences showed a homology with a putative Arabidopsis endoglycosidase gene product, however, some sequences were not found in the deduced amino acid sequence, indicating that the plant endoglycosidase would have diversity in the encoded genes.

Structural analysis of free *N*-glycans occurring in the rice cultured cells showed that the high-mannose type free *N*-glycans ($Man_{9\text{-}5}GlcNAc_1$) occur mainly in the soluble fraction at concentration of 140.1 nmol/g of wet cells, showing the endo-OS functions in the cultured cells to produce the free *N*-glycans. In contrast, the amount of the plant complex type free glycans (1.2 nmol/g of wet cells) was much lower than that of high-mannose type ones, indicating that a PNGase might be involved in turn over of matured glycoproteins . To confirm whether matured glycoproteins expressed in the rice cultured cells bear the high-mannose type glycans as endogenous substrates for endo-OS, we analyzed the structures of conjugated *N*-glycans. Glycopeptides were prepared from actinase digests of water-soluble and water-insoluble proteins in the rice cultured cells. *N*-glycans were released from the glycopeptides, which were prepared by the actinase digestion from water-soluble and water-insoluble glycoproteins, by hydrazinolysis. The resulting sugar chains were *N*-acetylated and pyridylaminated. The structures of PA-sugar chains were identified by 2D-sugar chain map, exo-glycosidase digestion and ESI-MS analysis. Major five structures of the *N*-glycans identified belonged to the plant complex type structure having a β-1,2-xylose and an α-1,3-fucose residues. On the contrary, the high mannose-type structures could not been found in relevant amount among the total sugar chains of rice glycoproteins. These results seems to suggest that the endogenous substrates for the enzyme could not be matured glycoproteins but denatured glycoproteins or glycopeptides produced in the ERAD system.

(236) **Acceptor Specificity of a Galactofuranosyltransferase from *Trypanosoma cruzi***

Wagner B. Dias[1], Carla V. Loureiro y Penha[2], Murielle Girard[1], Orlando A. Agrellos[1], Thomas N. Oeltmann[3], Norton Heise[1], Jose O. Previato[1] and Lucia MendonÁa-Previato[1]

[1] Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21949-900, Cidade Universit·ria, Rio de Janeiro, RJ, Brasil, [2] Departamento de Biologia Celular e GenÈtica, Universidade do Estado do Rio de Janeiro, 20550-900, Rio de Janeiro, RJ, Brasil, [3] Department of Biology, Vanderbilt University, Nashville, TN 37235, USA.

Galactofuranosyltransferases (Gal*f*-T) are glycosyltransferases which transfer D-galactofuranosyl (Gal*f*) residues from UDP-Gal*f* to their specific positions in a variety of surface polymers, such as arabinogalactan of mycobacterial cell walls, bacterial *O*-antigens, fungal and protozoan glycolipids and glycoproteins. While in mycobacteria, Gal*f* residues are essential for the cell wall assembly, in *Leishmania* spp. an internal Gal*f* residue is an integral part of the core structure of lipophosphoglycan (LPG) and thus necessary for the surface expression of this parasite virulence factor. Depending on the species of *Leishmania*, terminal Gal*f* residues can also be found in surface glycoinositolphospholipids (GIPLs). In

Trypanosoma cruzi Gal*f* residues are found in GIPLs and cell-surface glycoproteins like the GP72 and the *O*-linked oligossacharide chains of mucin-like glycoproteins. The occurrence of Gal*f* residues in pivotal structures of pathogenic microorganisms and their absence in humans make both the UDP-galactosyl mutase and Gal*f*-T enzymes attractive targets for new chemotherapeutic agents. In order to identify and partially characterize the substrate specificity of the *T. cruzi* Gal*f*-T, we developed an assay using the an extract of *Escherichia coli* overexpressing the recombinant UDP-galactosyl mutase of *Mycobacterium smegmatis* to generate the UDP-Gal*f* *in situ* (Mikusova et al., 2000, JBC: 275, 33890), *T. cruzi* microsomal membranes as source of Gal*f*-T activity, and 3 highly purified mannosylinositolphosphoryl-ceramides (MIPC), obtained from yeast forms of *Sporothrix schenckii*, as acceptor substrates. These MIPC substrates included Man*p*(α1→3)Man*p*(α1→6)IPC (M_2IPC) and two related compounds containing Man*p*(α1→6)Man*p*(α1→ (M_4IPC) and Man*p*(α1→2)Man*p*(α1→6)Man*p*(α1→ (M_5IPC) linked to the *O*-3 position of the terminal mannose of M_2IPC. The results clearly showed that *T. cruzi* Gal*f*-T is able to transfer Gal*f* residues from UDP-Gal*f* to the M_5IPC but not to M_2IPC or M_4IPC. The ability of *T. cruzi* Gal*f*-T to elongate only the M_5IPC indicates that this enzyme is able to discriminate terminal Man*p*(α1→2)Man*p* from Man*p*(α1→3)Man*p* and Man*p*(α1→6)Man*p* substructures. Supported by: CNPq, FAPERJ, IFS, and John Simon Guggenheim Memorial Foundation.

(237) **UDP-*N*-acetylglucosamine:polypeptide *N*-acetylglucosaminyltransferase (ppGlcNAcT) from *Crithidia fasciculata*.**

Ana Paula S.A. Santiago[1], Lucia MendonÁa-Previato[1], Norton Heise[1], Orlando A. Agrellos[1], Christopher M. West[2] and Jose O. Previato[1]

[1] Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21949-900, Cidade Universit·ria, Rio de Janeiro, RJ, Brasil, [2] University of Oklahoma Health Sciences Center, Dept. Biochemistry & Molecular Biology, BMS 937 P.O.Box 26901, Oklahoma City OK 73190.

Threonine *O*-glycosylation in *Trypanosoma cruzi* and *Dictyostelium discoideum* is mediated by a Golgi ppGlcNAcT enzyme that catalyzes the transfer of GlcNAc from UDP-GlcNAc to clustered Thr residues, near proline, of surface glycoproteins. It has been suggested that this unusual glycosylation seen in lower eukaryotes might have substituted for the GalNAcα Thr/Ser linkage of animals before the epimerase, that converts UDP-GlcNAc into UDP-GalNAc, had evolved. In order to gain insight into a possible evolutionary history of Thr glycosylation by GlcNAc, four peptides were screened to detect the ppGlcNAcT in *Crithidia fasciculata*. The results showed that *C. fasciculata* microsomal membranes transfer GlcNAc residues to peptides used to measure addition of α GlcNAc to the mucin-like domain of the protein SP29/PsA (TVTPTVTPTVTPTPTNTPNPTPSQTS/rPsAT and TVTPSVSPTVTPTPTNTPNPTPSQTS/rPsAS), the spore coat protein SP85/PsB (TYPPTQPPTQPPTYPP/T16), each from *D. discoideum*; and the Thr-rich dodecapeptide encoded by *T. cruzi* MUC gene (KPPTTTTTTTTKPP/KPT). Incorporation of GlcNAc is dependent on incubation time, concentration of enzyme and peptide substrates. Although always linear, GlcNAc incorporation varies according to the peptide used. The following order of substrate preferences were: rPsAT>rPsAS>T16>KPT, in a ratio of 16>14>8>1, respectively. Results showed that the *C. fasciculata* ppGlcNAcT preferred *D. discoideum* protein-derived peptides as acceptors. It is worth mentioning that the existence of *O*-GlcNAc-modified proteins in *C. fasciculata* has yet to be reported. Nevertheless, the occurrence of ppGlcNAcT was confirmed by characterization of the glycosylated peptide product. Studies to characterize the *C. fasciculata* GlcNAc-native peptide acceptor are under way. Supported by: CNPq, FAPERJ, and John Simon Guggenheim Memorial Foundation.

(238) **Purification and Some Properties of a Cobalt-Sensitive α-Mannosidase from Ginkgo Biloba Seeds**

Kwan Kit Woo[1] and Yoshinobu Kimura[1,2]

[1] The Graduate School of Natural Science and Technology, Okayama University. ,
[2] Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University.

We have already reported that free *N*-glycans occur in various plant cells, especially in developing or growing cells. If such free *N*-glycans play a critical role as one of signaling molecules during plant cell development or growth, it is reasonable to assume that α-mannosidase(s) should be involved in the turnover of the putative bioactive oligosaccharides. Concerning the plant α-mannosidase, many acidic α-mannosidases have been purified and characterized from seeds and fruits so far. From the view point of physiological function of the α-mannosidase of plant cells, however, these plant enzymes, which consist of two heterogenous subunits, have been considered to be involved in the degradation of *N*-glycosylated proteins in the vacuole or protein body. Therefore, we started to purify and characterize an α-mannosidase involved in the degradation of the free *N*-glycans occurring in the plant cells.

In this study, we have purified an α-mannosidase from *Ginkgo biloba* seed, of which the molecular weight was estimated to be 120 kDa by SDS-PAGE in the presence of 2-mercaptoethanol, and 340 kDa by gel filtration, indicating that the enzyme may function in oligomeric structure in the plant cell. The α-mannosidase showed optimum activity at weak acidic pH region (pH 5.0). The *N*-terminal amino acid sequence was Ala-Phe-Met-Lys-Tyr-X-Thr-Thr-Gly-Gly-Pro-Val-Ala-Gly-Lys-Ile-Asn-Val-His-Leu-. The α-mannosidase activity for Man5GlcNAc1 was enhanced by an addition of Co^{2+}however, addition of Zn^{2+}, Ca^{2+}, and EDTA did not show any significant effect. In the presence of cobalt ion, the hydrolysis rate for Man6GlcNAc1-PA was significantly faster than that for Man6GlcNAc2-PA, suggesting that the enzyme would be involved in the degradation of free *N*-glycans occurring in the developing plant cells [Kimura, Y., & Matsuo, S. (2000) J. Biochem. 127, 1013-1019]. The Ginkgo α-mannosidase activated with Co^{2+} hydrolyzed the α-1,2-Man, α-1,6-Man, and α-1,3-Man bonds from the non-reducing termini of M9A to lead to Manβ1-4GlcNAc-PA. On the other hand, in the absence of Co^{2+}, the enzyme showed an α-1,2- mannosidase-like activity, suggesting that Co^{2+} may alter the substrate specificity as well as the hydrolysis rate of the enzyme. The hydrolytic pathway from M9A to M1 derived by the cobalt-activated enzyme will be discussed in detail.

(239) **Glycoinositolphosphosphingolipids from Eringi Mushroom**

Saki Itonori[1], Akiyo Uda[1], Kouji Yano[1], Noriyasu Hada[2], Tadahiro Takeda[2] and Mutsumi Sugita[1]

[1] Faculty of Liberal Arts and Education, Shiga University, 2-5-1 Hiratsu, Otsu, Shiga 520-0862, Japan,
[2] Kyoritsu College of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan.

Edible fungi, mushrooms, are a popular food in Japan and over ten cultured mushroom species are available in Japanese food markets. Recently, constituents or ingredients of edible mushrooms have drawn attention because possibilites have been seen for their medical usage. Especially, in the Agaricus, β-glucan or ergosterol may have immunoenhancing and antitumor activities. Glycosphingolipids of the edible fungi have been reported as glycosylceramide (cerebroside) with 9-methyl-4,8-octadecasphingadienine or trihydroxysphinganine (phytosphingosine) and 2-hydroxy fatty acid. These unique ceramide constituents are common structures in plants, but differ from those in animals. Basidiolipids (mycoglycolipids) of higher mushrooms have been characterized as glycosylinositolphosphoceramides, and these have a common core structure of Manα1-2Ins1-[PO_4]-Cer and extension of Man, Gal and/or Fuc sugar moieties.

In our laboratory, mycoglycolipids were isolated from the Eringi mushroom, *Pleurotus eryngii* (engl. king oyster mushroom) by successive column chromatography on ion exchange Sephadex (DEAE-Sephadex), and silicic acid (Iatrobeads). Their structures were characterized by gas-liquid chromatography (GC), gas chromatograph-mass spectrometry (GC-MS), matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and ^{1}H-NMR spectrometer.

Ceramide constituents of these mycoglycolipids were composed of phytosphingosine and C16 to C24 fatty acid or a long chain 2-hydroxy fatty acid. Mycoglycolipids from Eringi mushroom have a common core structure with extensions of galactose and branched galactose and fucose. Carbohydrate constituents were composed as follows; AGL1, Ins:Man=1:1, AGL4, Ins:Man:Gal:Fuc=1:1:2:1, AGL5, Ins:Man:Gal:Fuc=1:1:3:1. For determination of linkage between Man and Ins, the inositol periodate oxidation fragment was identified as erythritol by GC and GC-MS. Partial methylated alditol acetate derivatives from AGL4 and AGL5 were detected as 1,2,6-substituted Gal and 1,2,3,6-subustituted Gal, respectively. After mild acid hydrolysis and sequential α- and β-galactosidase treatments, the chemical structures of these mycoglycolipids were characterized as Ins1-[PO_4]-Cer (AGL0), Manα1-2Ins1-[PO_4]-Cer (AGL1), Galα1-6(Fucα1-

2)Galβ1-6Manα1-2Ins1-[PO_4]-Cer (AGL4), and Galα1-6(Galα1-3)(Fucα1-2)Galβ1-6Manα1-2Ins1-[PO_4]-Cer (AGL5).

(240) **β-Glucuronosyltransferase from Radish Primary Roots Involved in the Synthesis of Arabinogalactan-proteins**

Maya Endo[1], Tomoyuki Konishi[1], Toshihisa Kotake[1], Kazumasa Kimura[2] and Yoichi Tsumuraya[1]

[1] Department of Biochemistry and Molecular Biology, Faculty of Science, Saitama University, 255 Shimo-okubo, Saitama 338-8570, Japan, [2] Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi-shi, Tokyo 186-8650, Japan.

Arabinogalactan-proteins (AGPs) are a class of proteoglycans found in higher plants. They appear to be involved in various processes including cell proliferation, cell expansion, somatic embryogenesis, and cell death. The basic carbohydrate structure of AGPs comprises a β-(1→3)-galactan, branched with consecutive (1→6)-linked β-Gal residues, to which glucuronic acid (GlcA) and/or 4-*O*-methyl-GlcA residues are attached through β-(1→6)-linkages at nonreducing termini. The β-Gal side chains are also usually heavily substituted with α-L-Ara*f* residues. We have studied the properties of a β-glucuronosyltransferase (GlcAT) that is involved in the synthesis of the carbohydrate portion of AGPs.

The reaction was carried out in a mixture composed of a microsome fraction prepared from 6-d-old primary roots of radish (*Raphanus sativus* L. var *hortensis*), 2 mM UDP-[^{14}C]GlcA, 5 mg/ml β-(1→3)-galactan as an acceptor, 30 mM $MnCl_2$, 0.75% Triton X-100, and 40 mM MES-KOH buffer (pH 6.0) at 25˚C for 10-120 min. The GlcA transfer occurred maximally at pH 6.0, in the presence of 30 mM Mn^{2+} and 0.75% Triton X-100. The specific activity of GlcAT was in the range of 50-150 pmol/min/mg protein, while activity to endogenous acceptors was very low. The transfer reaction for an AGP from radish mature roots was insignificant but could be increased by prior enzymatic removal of α-L-Ara*f* residues or both α-L-Ara*f* residues and (1→6)-linked β-Gal side chains by digestion with α-L-arabinofuranosidase or both α-L-arabinofuranosidase and endo-β-(1→6)-galactanase [1], respectively. Digestion of the radiolabeled products formed from β-(1→3)-galactan with exo-β-(1→3)-galactanase released mainly radioactive β-GlcA-(1→6)-Gal and β-GlcA-(1→6)-β-Gal-(1→6)-Gal. This was also the case for the transfer product formed from the modified AGP. The structure of these acidic oligosaccharides was verified by digestion with β-glucuronidase [2] purified from *Aspergillus niger*. These results indicate that the transfer of [^{14}C]GlcA occurred preferentially onto consecutive (1→3)-linked β-Gal chains as well as single branched β-(1→6)-Gal residues through β-(1→6)-linkages, resulting in the formation of branched acidic chains. The structural feature of the transfer products is consistent with that found in various AGPs.

The enzyme also could transfer [^{14}C]GlcA residues onto several oligosaccharides including β-(1→6)- and β-(1→3)-galactotrioses and α-L-Ara*f*-(1→3)-β-Gal-(1→6)-Gal. A branched tetramer, α-L-Ara*f*-(1→3)-[β-GlcA-(1→6)-]β-Gal-(1→6)-Gal, was formed from the last oligosaccharide acceptor, suggesting that the α-L-Ara*f* residues substituted at nonreducing termini of (1→6)-linked β-Gal side chains do not interfere with the transfer of GlcA residues onto the β-Gal residues.

[1] Okemoto et al., *Carbohydr. Res*., 338 (2003) 219-230
[2] Kuroyama et al., *Carbohydr. Res*., 333 (2001) 27-39

(241) ***N*-Glycans bearing β-1,3-galactosyl residue in Royal Jelly Glycoproteins.**

Masaki Miyamoto[1], Kazunori Tsumura[1], Mariko Kimura[2], Seiji Okihara[3], Hiroyuki Sugimoto[3], Hideo Yamada[3] and Yoshinobu Kimura[1,4]

[1] 1) The Graduate School of Natural Science and Technology, Okayama University. , [2] Department of Food System, Kurashiki Sakuyo University., [3] Yamada Apiculture Center Inc. , [4] Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University.

Recently , we revealed that a β-1,3-galactosyl residue occurs in *N* -glycans of royal jelly glycoproteins. [Kimura, Y., *et al., Biosci. Biotechnol. Biochem.*, 67, 1852-1856 (2003)]. In the previous report, we have determined two kinds of structures having the β-1,3-Gal, one was complex type and the other one was hybrid type structure. Our results clearly showed that a β-1,3-galactosyl transferase should be involved in the *N* -glycan processing in honeybee cells. In this study, we have found another complex type structures bearing two galactosyl residues or two GalNAc residues among the *N* -glycans linked to the insect glycoproteins. Furthermore, we identified the glycoprotein bearing the Gal-containing *N* -glycans and determined the glycosylation site. The glycopeptides having the Gal-containing residue were purified from a tryptic digest of royal jelly glycoproteins by a combination of Con A and PNA affinity chromatography and RP-HPLC. The amino acid sequence of the purified glycopeptide was G-E-S-L-N(CHO)-K, corresponding to Gly (24) to Lys(29) of one of major royal jelly glycoprotein (MRJ1). The *N*-glycans were liberated from the glycopeptides by hydrazinolysis. After *N*-acetylation, the sugar chains were coupled with 2-aminopyridine. The PA-sugar chains were purified by RP-HPLC and SF-HPLC. The structures of the PA-sugar chains were determined by a combination of exoglycosidase and endoglycosidase digestions, ESI-MS, methylation analysis, and 1H-NMR. The *N*-glycans purified the PNA-bound glycopeptides were revealed to contain β-1,3-Gal or β-1,4-GalNAc residues. From these structures determined, we could deduce a new processing pathway for *N*-glycans in honeybee cells, suggesting that the *N*-glycosylation mechanism in the insect cells would be slightly different from those in plant and animal cells.

(242) **Evaluation of the Recombinant α-galactosidase A Produced in Yeast Toward Cultured Fibroblast Cells from Fabry Patients and Fabry Model Mice**

Yasunori Chiba[1], Hitoshi Sakuraba[2], Yuki Takaoka[1], Minako Takashiba[1], Yoshiko Kasahara[1], Kazuo Kobayashi[3] and Yoshifumi Jigami[1]

[1] Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology, [2] Department of Clinical Genetics, The Tokyo Metropolitan Institute of Medical Science, [3] CMC R&D Laboratory, Pharmaceutical Division, KIRIN Brewery Co., Ltd..

Fabry disease is an X-linked inborn error of glycolipid metabolism caused by deficiency of the lysosomal α-galactosidase. In order to develop an effective and economical enzyme replacement therapy for Fabry disease, we produced α-galactosidase A in budding yeast *Saccharomyces cerevisiae*. The recombinant α-galactosidase A was purified from 150 L cultural supernatant of the yeast strain that contained highly phosphorylated sugar chains by a successive separation through the anion-exchange, hydrophobic-interaction and gel filtration chromatography. Its purity was judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase column chromatography. The molecular mass was determined as 45 kDa by SDS-PAGE and MALDI-TOF mass spectrometry. The monosaccharide content of recombinant α-galactosidase A was determined by the method that combined a laser induced fluorescence detection of APTS-labeled monosaccharide with capillary electrophoresis. The recombinant enzyme contained 3.8 mol of Man-6-P residues per dimer of α-galactosidase A, the value of which was a little higher than that of Fabrazyme prepared from recombinant CHO cells by the other group. The recombinant enzyme, which was treated with newly isolated α-mannosidase to expose the mannose-6-phosphate residues in the sugar chain, was added into the medium at the concentration of 0.5-3 μg/ml. Dose-dependent uptake of the enzyme and hydrolysis of the accumulated ceramide trihexoside (CTH) were observed in the fibroblast from Fabry patient. The uptake was inhibited by 5 mM Man-6-P, indicating that incorporation of the enzyme was dependent on Man-6-P receptor in the cell surface. To evaluate the recombinant α-galactosidase A for the replacement therapy, we injected single or repeated doses of the enzyme at 3 mg/kg body weight to Fabry mice. Normal or greater enzyme activity was detected in various tissues of Fabry model mice by single dose injection, and repeated doses of the enzyme injection caused a decrease of the accumulated CTH in liver, kidney and heart. We also tried to express the α-galactosiase A in methylotrophic yeast *Ogataea minuta*. Productivity and properties of the recombinant α-galactosidase A purified from *O. minuta* will be discussed.

(243) **A Single Amino Acid at the C-terminus of *Helicobacter pylori* α1,3/4 Fucosyltransferase Determines Substrate Specificity**

Bing Ma[1], Monica M Palcic[2], Bart Hazes[1] and Diane E Taylor[1]

[1] Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta
T6G 2H7, Canada, [2] Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada.

Fucosyltransferases (FucT) from different *H. pylori* strains display distinct Type I (βGal1,3βGlcNAc) or Type II (βGal1,4βGlcNAc) substrate specificity. FucT from *H. pylori* strain NCTC11639 is only able to transfer fucose to the OH-3 of Type II substrate to synthesize Lewis X, thus it has exclusive α1,3 activity. FucT from *H. pylori* strain UA948 is able to transfer fucose to the OH-3 of Type II acceptor to produce Lewis X, as well as to the OH-4 of Type I acceptor to form Lewis A, so it has both α1,3 and α1,4 activities. Our domain swapping study (Ma *et al*., J Biol. Chem. 2003, 278: 21893-21900) demonstrated that exchange of residues in the C-

terminal hypervariable region, specifically, seven amino acids (^{347}DNPFIFC^{353}) in 11639FucT and ten amino acids (^{345}CNDAHYSALH^{354}) in UA948FucT, was sufficient to either confer or abolish α1,4 activity. Our current investigation aims to identify which amino acids within ^{345}CNDAHYSALH^{354} of UA948FucT are responsible for conferring Type I substrate specificity. Alanine scanning site-directed mutagenesis was performed whereby each non-alanine residue within ^{345}CNDAHYSALH^{354} of UA948FucT was mutated to alanine. The ability of each mutant to transfer fucose to Type I and Type II acceptors was characterized. The Tyr^{350}→Ala mutation dramatically reduced α1,4 activity to 1.8% of the wild type (WT) level, whereas the α1,3 activity was maintained at a similar level to that of WT. None of the other single amino acid mutations selectively modified α1,4 activity. These results suggested that Tyr^{350} in UA948FucT is responsible for conferring Type I substrate specificity. Sequence alignments between 11639FucT and UA948FucT showed Phe^{350} or Phe^{352} from 11639FucT may be aligned with Tyr^{350} in UA948FucT. To determine whether the hydroxyl group of Tyr^{350} plays an essential role in Type I acceptor specificity, a Tyr^{350}→Phe mutation was made in UA948FucT. This mutant exhibited decreased levels of both α1,3 and α1,4 activities, nevertheless the α1,4/α1,3 ratio was similar to that of WT, indicating that the hydroxyl group in Tyr^{350} was not critical for Type I acceptor specificity. On the other hand, 11639FucT Phe^{350}→Tyr and 11639FucT Phe^{352}→Tyr mutants did not acquire any α1,4 activity, implying that Tyr residue itself was not sufficient to confer α1,4 activity. Our data demonstrates that a single tyrosine residue in the C-terminal hypervariable region of *H. pylori* UA948FucT determines Type I acceptor specificity. In mammalian α1,3/4 FucTs, Type I acceptor preference is also determined by a single aromatic amino acid (Trp), but it is located in the N-terminal hypervariable stem domain.

(244) **Critical Elements of Oligosaccharide Acceptor Substrates for the *Pasteurella Multocida* Hyaluronan Synthase**

Kellie J. Williams[1], F. Michael Haller[2], Johannis P. Kamerling[3] and Paul L. DeAngelis[1]

[1] Univ. of Oklahoma Health Sciences Center, Dept. of Biochem. and Molec. Biol., Oklahoma Center for Medical Glycobiology, 940 S.L. Young, Oklahoma City, OK 73104, USA, [2] Hyalose L.L.C., 655 Research Parkway, Suite 525, Oklahoma City, OK 73104, USA, [3] Bijvoet Center for Biomolecular Research, Department of Bio-Organic Chemistry, Section of Glycoscience and Biocatalysis, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands.

Glycosaminoglycans (GAGs) are an essential class of heteropolysaccharides that includes hyaluronan (HA), heparin, and chondroitin. These molecules serve important roles in vertebrates including intracellular adhesives, signaling molecules, lubricants, anticoagulants, and structural elements in connective tissue. Certain bacteria produce extracellular GAG capsules to avoid host defenses and increase virulence. Dual-action glycosyltransferases known as synthases produce the repeating disaccharide units of the GAG chain. Presently, no synthase crystal structures are available and only minimal information on the critical molecular elements of the enzymes are known. Our work focuses on the bacterial *Pasteurella multocida* hyaluronan synthase that catalyzes the polymerization of hyaluronan chains. In order to analyze the synthase's intrinsic mechanisms, we have tested a variety of sugar analogs for their ability to serve as acceptors in elongation assays. The relative affinity (Michaelis constants, K_M) and the relative activity of a series of authentic, synthetic hyaluronan ($[\beta4GlcUA\text{-}\beta3GlcNAc]_n$) = $[AN]_n$) oligosaccharides containing a methoxyphenol (MP) group at the non-reducing termini were analyzed. The sugars tested included AN-MP, ANA-MP, ANAN-MP, ANANAN-MP, N-MP, NA-MP, NAN-MP, NANA-MP, and NANAN-MP. Chemically modified analogs with a variety of aglycones and substituents were also tested. Our approach has enabled us: (1) to determine the optimal length of the sugar polymer necessary for efficient chain elongation, (2) to identify a potential role for the acyl group of the hexosamine, and (3) to design a synthetic, artificial acceptor for the synthase enzyme. These analogs also provide clues to the nature of the HA synthase active site. We hypothesize that the size of the enzyme's acceptor binding pocket corresponds to the size of the smallest high affinity substrate. Characterization of acceptor analogs with varied acyl chains also suggests a role for the hydrophobic interaction in sugar binding. Overall, this information advances the knowledge of GAG biosynthesis as well as assists the creation of various therapeutic sugars for medical applications in the future. (Supported by National Science Foundation and Oklahoma Center for Advancement of Science and Technology)

(245) **Towards the Understanding of the Catalytic Mechanism and Substrate Specificities of Sialyltransferases from *Campylobacter jejuni***

Cecilia P.C. Chiu[1], Michel Gilbert[2], Luke L. Lairson[3], Andrew Watts[3], Warren W. Wakarchuk[2], Stephen G. Withers[3] and Natalie C.J. Strynadka[1]

[1] Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada, V6T 1Z3. , [2] Institute for Biological Sciences, National Research Council of Canada, Ottawa, ON, Canada, K1A 0R6., [3] Department of Chemistry, University of British Columbia, Vancouver, BC, Canada, V6T 1Z1..

Sialic acid is considered one of the most critical carbohydrates in cell biology due to its presence on the surface of all mammalian cells. It is involved in numerous cellular activities such as general adhesion and recognition, and may play a role in diseases such as leukemia and cancer. It is also found on the outer membrane-bound lipooligosaccharides and capsular polysaccharides of a number of bacterial pathogens including *Campylobacter jejuni, Neisseria gonorrhoeae, N. meningitidis, Haemophilus influenzae, Pasturella (Mannheimia) haemolytica and Escherichia coli*. The presences of these sialylated-glycoconguates have been correlated with the pathogenesis of these bacteria. The enzymes which transfer the sialic acid moiety to the peripheral glycoconjugates are known as sialyltransferases. Several versions of sialyltransferase (Cst's) are found in *C. jejuni*, our model system. These enzymes are involved in synthesis of sialylated-lipooligosaccharides that mimic human gangliosides. This mimcry has been correlated with the auto-immune neuropathies Guillain-BarrÈ syndrome and Miller Fisher syndrome that develop after some *C. jejuni* infections. We have previously reported the structure of a bifunctional alpha-2,3/8-sialyltransferase CstII, which catalyzes the transfer of sialic acid from CMP-sialic acid onto Gal-β-1,3-GalNAc at position 3 and subsequently onto position 8 of the product of the first reaction, namely NeuAc-α-2,3-Gal-β-1,3-GalNAc. The structure of the enzyme with a bound donor analogue, CMP-3-fluoro-NeuAc suggests that His188 acts as the general base in the reaction. Asn51, one of the amino acids that directly interacts with the carboxyl group of the sialic acid, has been suggested to be the key residue that governs the switch between mono- and bifuncationality of the enzyme, as an Asn51Thr mutation abolishes alpha-2,8 activity. Recently we obtained the complex structure of CstI, a monofunctional version also from *C. jejuni*, with CMP-3F-NeuAc. CstI shares 53% sequence identity with CstII, however, it is only capable of catalyzing the alpha-2,3 transfer. Interestingly, CstI also has an asparagine (Asn66) in the same Asn51 position of CstII. The overall fold of CstI is almost identical to CstII (with rms deviation of 0.7≈ between 214/246 Cα atoms), with the most significant differences in the loop region that folds over the active site. These rather subtle differences give clues as to the diverse substrate specificities of the two enzymes. Co-crystallization of either one of the enzymes with both donor sugar acceptor sugars is underway in order to obtain a clearer picture of the reaction mechanism as well as the different acceptor binding modes of the two enzymes. Structure based inhibitors can be designed for these enzymes as they represent attractive anti-bacterial targets against these human pathogens.

(246) **Isolation and Characterization of Mannoprotein-releasing Mutant of *Saccharomyces cerevisiae***

Miwa Tanaka[1], Tetsuji Odani[1], Toshifumi Yuuki[1], Yoh-ichi Shimma[2], Takehiko Yoko-o[2], Yasunori Chiba[2], Yasuyuki Ohtake[1] and Yoshifumi Jigami[2]

[1] Fundamental Research Laboratory, Asahi Breweries, Ltd., 1-1-21, Midori, Moriya-shi, Ibaraki, 302-0106, JAPAN, [2] Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology, AIST Central 6, Tsukuba, Ibaraki, 305-8566, Japan .

The cell wall of yeast is composed of glucan, mannoprotein, and chitin. It has been reported that mannoproteins, extracted by alkaline treatment from cell wall fraction of cropped brewing yeast, are more potent than β-glucans in improving gut health and enhancing systemic immune-function. However, since the amount of cropped brewing yeast depends heavily on production of beer, we attempted to obtain mannoproteins by a single-step fermentation system. Here we report the isolation of the novel mutants that release mannoproteins into the culture medium. It is possible that mannoproteins recovered from the medium may have different functions from conventional mannans because the peptide bond and *O*-glycosydic bond will not be cleaved due to the lack of chemical treatment.

Several mannoprotein-releasing mutants were isolated by ethyl methane sulfonate mutagenesis using *Saccharomyces cerevisiae* strain YNN27. The strain named MTY-9, was selected for further study because it released

stably a large quantity of mannoproteins among them. MTY-9 cells showed a slower growth, larger and rounder shape than wild-type YNN27 cells, and aggregation in liquid medium. The mannose/glucose ratio of cell wall was about 0.5 in MTY-9 cells, while that was about 1.0 in wlld-type cells, indicating that MTY-9 cell wall contains lesser mannose than wild-type cell wall. MTY-9 was back-crossed three times with wild-type strain. Diploids were sporulated and dissected. Four ascospores showed the 2:2 segregation patterns of mannoprotein-releasing phenotype expected for a single gene mutation.

To identify the corresponding gene, MTY-9 cells were transformed with the plasmid containing a genomic DNA library. The plasmid recovered from the transformants that showed wild-type phenotypes was sequenced for its insert DNA fragment in order to identify the responsible ORFs. The identified ORF was *GPI10*, which encodes an integral membrane protein of 616 amino acids. *GPI10* is essential for the viability of yeasts and the gene product is predicted as a putative α-1, 2-mannosyltransferase to add the third mannose onto the glycosylphosphatidylinositol (GPI) core structure. Sequencing of *GPI10* and its flanking regions of MTY-9 revealed the presence of a single missense mutation consisting of a C to T transition, which changes Pro498 into Leu.

We are currently performing the identification of mannoprotein species released into the culture medium and structural analysis of their oligosaccharides.

(247) **Large Scale Preparation and Characterization of Yeast Mannoproteins**

Tetsuji Odani[1], Miwa Tanaka[1], Toshifumi Yuuki[1], Masaaki Yasue[1] and Yoshifumi Jigami[2]

[1] Fundamental Research Laboratory, Asahi Breweries, Ltd., 1-1-21, Midori, Moriya-shi, Ibaraki, 302-0106, JAPAN, [2] Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology, AIST Central 6, Tsukuba, Ibaraki, 305-8566, Japan.

Mannoproteins are one of the major components of yeast cell wall. We have found that among yeast cell wall components, mannoproteins are more potent than β-glucans in improving gut health and enhancing systemic immune-function. We, as Breweries, had been tried to recover mannoproteins from cropped yeast, however, purification of the mannoproteins from cropped yeast includes wash and extraction steps by alkaline, which are time and cost consuming with a poor recovery. Thus, the development of effective method to recover mannoproteins is essential to utilize this material as a dietary supplement or functional food.

To develop a novel method to prepare bulk mannoproteins from baker's yeast, we constructed a mutant yeast diploid cell (AB9) that is derived from a several cross of different mating type cells containing a MTY9 derived original mutation of *GPI10* and releases mannoproteins from its cell wall into culture medium. We present here a simple method to recover a large amount of mannoproteins from a supernatant of the yeast culture.

Yeast cultivation using 5L jar fermentor was carried out under the commonly used conditions, such as 30 degrees C, pH 5.5, 1 vvm aeration and 200 rpm agitation. As the cultivation medium, we tested corn steep liquor (CSL)-glucose medium and modified defined mineral medium (MDMM, van Hoek *et al*, 2000, *Biotech. and Bioeng*., **68**, 517-522). Either media was suitable for the recovery of the mannoproteins, but, for the easy handling, we selected MDMM to cultivate this mutant. The supernatant of the culture was obtained by centrifugation and filtration to remove yeast cells completely. The mannoproteins could be recovered by two methods, ethanol precipitation and ultra filtration.

We adapted these conditions for 700L scale cultivation. After 44 hours cultivation, the supernatant was recovered and subjected to ultra filtration to concentrate mannoproteins and to wash out smaller molecules including glucose polymers and salts. Mannoprotein sample was then obtained by freeze-drying or spray drying from this concentrated fraction. The amount of recovered mannoprotein sample was about 350g.

The mannoprotein sample was lightly yellow colored, tasteless, odorless and water soluble powder. Total sugar content was about 85%, containing more than 99.5% of mannose and a trace amount of glucose. Protein content was about 15% and its amino acid composition was characteristically serine and threonine rich. Average molecular weight was estimated to be more than 250 kDa by SDS-PAGE.

We are now evaluating the physiological function of the above mannoproteins *in vitro* and *in vivo*. On going results will be presented.

(248) **Investigation of Oligosaccharide Function in an Insect Hexamerin by Site-Directed Mutagenesis**

Ok-ki Cho[1], Han Ho Choi[2], San Mong Lee[3] and Soohyun Kim[1]

[1] Proteome Analysis Team, Korea Basic Science Institute, Daejeon 305-806, Korea, [2] Dept of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-806, Korea, [3] Dept of Sericultural and Entomological Biology, Miryang National University, Miryang 627-702, Korea.

Arylphorin is a kind of insect hexamerins present in hemolymph, which is composed of six identical subunits of 80 kDa. Mature arylphorin has four potential N-glycosylation sites but only two, second and third sites were practically glycosylated. Interestingly, monoglucosylated oligomannose type glycan ($Glc_1Man_9GlcNAc_2$) was existed exclusively at the second site and simple oligomannose and even truncated glycans were appeared at the third site. To investigate the role of N-glycosylation on arylphorin folding and assembly, N-glycosylation minus mutants were constructed by site-directed mutagenesis (each single or combined N196Q and N348Q) and expressed in E. coli and HEK293A (human embryonic kidney) cells. Arylphorin produced from E. coli transformed with wild type gene was almost recovered as inclusion body, although the protein moved the same distance as control arylphorin in SDS-PAGE. However, arylphorin was secreted to culture media from HEK293A cells during growth. Size of the subunit and native protein were well agreed with control arylphorin under reduced or non-reduced condition. Non-reduced arylphorin was migrated as a trimer in SDS-PAGE suggesting that arylphorin was comprised of two trimers bridged by inter-disulfide bond between subunits. Secondary structure was confirmed by circular dichroism spectrometry. All of these results demonstrated that appropriate N-glycosylation is necessary for arylphorin folding, assembly and traffic. Protein expression with mutant genes in HEK293A cells is on the way to confirm the structural and physiological role of the glycans. A series of experiments will be carried out for oligosaccharide profile and glycosylation site with the expressed proteins.

(249) **The Deletion Analysis of an α2,6-Sialyltransferase from a Marine Bacterium, *Photobacterium damsela* JT0160**

Hiroshi Tsukamoto, Yoshimitsu Takakura and Takeshi Yamamoto

Plant Innovation Center, Japan Tobacco Inc., 700 Higashibara, Iwata, Shizuoka 438-0802, JAPAN.

We previously purified and characterized an α2,6-sialyltrasferase from a marine bacterium, *Photobacterium damsela* JT0160 and cloned its gene. The purified enzyme has shown molecular mass of 64 kDa in gel filtration and 61 kDa in SDS-PAGE, suggesting that this enzyme is monomeric. An open reading frame (2,028 bp) of the gene encodes a protein composed of 675 amino acids with a predicted molecular weight of 76.5 kDa. The difference of the molecular mass between the purified enzyme and the protein encoded by the isolated gene suggests that this enzyme is processed during its maturation in *P. damsela*. Indeed, the N-terminal 15 amino acids are removed in the native purified enzyme and probably, processing at the C-terminal side would also occur. The amino acid sequence deduced from DNA sequence of the gene shows a multi-domain structure: the N-terminal hydrophobic domain, a probable signal peptide (Met-1 to Ala-15), the putative catalytic domain (Cys-16 to Ala-497) and the C-terminal domain (Asp-498 to Asp-675), a putative membrane-binding domain. The primary structure of the N-terminal region (Met-1 to Ala-497) of the enzyme is not homologous to other sialyltransferases in mammals and bacteria, such as *Campylobacter jejuni*, *Haemophilus ducreyi*, *Neisseria gonorrhoeae* and *Escherichia coli*. On the other hand, the amino acid sequence of the C-terminal domain (Asp-498 to Asp-675) has strong homology to those of the phosphate transport system regulatory proteins belonging to the Pho U family. Here, we show some deletion analyses of the enzyme to clarify the minimum unit of the catalytic domain. Interestingly, the enzyme lacking both N-terminal 110 amino acids and C-terminal 179 amino acids retained the activity transferring NeuAc from CMP-NeuAc to lactose. However, the enzyme lacking both N-terminal 230 amino acids and C-terminal 179 amino acids and the enzyme lacking C-terminal 261 amino acids resulted in the complete loss of the activity. This study provides some molecular insights into the core primary structure of catalytic domain in the α2,6-sialyltrasferase.

(250) **Functional Analysis of a Novel Bacterial *N*-linked Protein Glycosylation Pathway from *Campylobacter jejuni*.**

Paul G. Hitchen[1], Dennis Linton[2], Nick Dorrel[2], Saba Amber[3], Andrey V. Karlyshev[2], Howard R. Morris[1,4], Anne Dell[1],

Miguel A. Valvano[5], Markus Aebi[3] and Brendan W. Wren[2]
[1] Department of Biological Sciences, Imperial College London, SW7 2AY, UK., [2] Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK., [3] Institute of Microbiology, Department of Biology, Swiss Federal Institute of Technology, Z¸rich, CH-8092 Z¸rich, Switzerland., [4] M-SCAN Mass Spectrometry Research and Training Centre, Silwood Park, Ascot SL5 7PZ, UK., [5] Departments of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1, Canada..

Recently an *N*-linked protein glycosylation system was identified in the human enteropathogen *Campylobacter jejuni*, the first example of this modification in a species from the domain Bacteria. We exploited the ability of this locus to function in *E. coli* to demonstrate through mutational and structural analyses that variant glycan structures can be transferred onto protein. We describe mutation of individual genes from the *C. jejuni N*-linked glycosylation locus and the structural characterisation of the *N*-glycans produced by such mutants. Furthermore, structural data on these variant glycans allowed us to infer the role of individual glycosyltransferases in the biosynthesis of the heptasaccharide *N*-linked glycan. We also demonstrate that the putative oligosaccharyltransferase protein PglB, is able to transfer a variety of glycans onto *C. jejuni* proteins and also show that the *C. jejuni* and *E. coli*-derived pathways can interact in the biosynthesis of *N*-linked glycoproteins. These data provide a framework for understanding the process of *N*-linked glycosylation in Bacteria and for devising strategies to exploit this system for glycoengineering.

(251) **Isolation and Characterization of the Essential Trehalose-Containing Metabolite in *Mycobacterium smegmatis***

Peter J. Woodruff, Fiona Lin and Carolyn R. Bertozzi
Departments of Chemistry and Molecular and Cell Biology, University of California, Berkeley.

The nonreducing disaccharide trehalose is widely found in prokaryotes, where it usually serves as a general osmoprotectant and/or thermoprotectant. In addition to this role as an osmo/thermoprotectant, mycobacteria enzymatically modify trehalose, adding on sulfate groups, phosphate groups, or lipid chains. The majority of these modified trehalose-containing species are associated with the lipid-rich mycobacterial cell wall. There are over a dozen trehalose-containing glycolipids that have been characterized in the literature, including the immunologically significant cord factor and sulfolipid-1. Mycobacteria also possess three biosynthetic pathways to make the sugar from three different substrates. This is in noticeable contrast to other organisms, which typically only have one pathway. By disrupting the biosynthetic pathways that produce trehalose, our laboratory has demonstrated that trehalose is essential for *Mycobacterium smegmatis.* Since mycobacteria are commonly found with an array of trehalose-containing glycolipids and lipooligosaccharides, we are using several methods to determine which one could be the vital species. Using chemical analogs of trehalose, we are mapping the important positions of the sugar for *M. smegmatis.* In addition, we are conducting biochemical assays to isolate the essential trehalose-containing metabolite.

(252) **Structural Diversity of C. elegans Glycome**

Andy Hanneman and Vernon Reinhold
Center for Structural Biology, Department of Chemistry University of New Hampshire, Durham, NH 03824.

Characterization of the glycans released from *C. elegans* protein extracts has been an elusive endeavor for a number of years.[1-6] In this report we have tried to account for these disparities and be more inclusion and comprehensive by including both *N*-, and *O*-linked glycans. Our preliminary findings had identified the usual set of *N*-linked pauci- and high mannose glycans, but a new and novel set of *N*-glycans of much greater abundance only released by hydrazinolysis. This latter group of structures were shown to be resistant to endoglycosidases and not observed following release by traditional β elimination. Consistent with this observation they were highly core-branched, rich in fucose, and galactose, as well as an expected abundance of mannose and *N*-acetyl glucosamine. Sequential mass spectrometry (MS^n) studies revealed details that included single and doubly branched fucose with additional monomers pendant to each fucose. Non-reducing termini were shown to consist of multiple isobars resplendent with fucosylation.

N-linked core structures were unraveled by taking advantage of the facile cleavage between the core GlcNAcs in MS^2 coupled with comparing reduced and non-reduced product spectra. Using this approach reducing-end fragments were unambiguously revealed by a +16 Da mass shift, identifying a set of structures incremented with one and two fucose moieties, each of which were shown to be terminated with a hexose residues. These latter termini were shown to be galactose residues linked β1-4 to the core 6-linked fucose, as confirmed by β-galactosidase treatment. One of these structures has been previously identified in the keyhole limpet[7,8]. The complementary reducing and non-reducing end fragments from isomeric glycans were subsequently followed in MS^{3-6}.

Beta elimination also indicated novel and heterogeneous structures among *O*-glycans at much lower levels, which also showed extensive fucosylation. Confirmation of this structural diversity has been expedited using unique bio-informatics tools developed in our laboratory. In support of these efforts we have been compiling an MS^n spectral library for use in identifying methylated glycan fragments, which are serving as searchable 'fingerprints' of glycan substructures. The identification of these novel glycan structures adds considerable complexity to the current understanding of protein glycosylation and the apparent normal phenotype when pathways to complex and hybrid glycans are eliminated.[9]

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(253) **Carbohydrate Distribution Pattern in *Caenorhabditis elegans* Using FITC-labeled Lectins**

Emerson Soares Bernardes[1], Daniela Smocking Rosa[2], Carlos Eduardo Winter[3], Maria Cristina Roque-Barreira[1] and JosÈ CÈsar Rosa[1,2]
[1] Dept. of Cell and Molecular Biology , [2] Protein Chemistry Center of Faculty of Medicine of Ribeir,,o Preto, University of Sao Paulo, [3] Dept. of Parasitology, Instituto de Ci'ncias BiomÈdicas, Unversity of Sao Paulo.

Development of multicellular organism is a process that involves cell fate determination, cell differentiation and cell communication. The whole process is regulated by several complex networks of genes, which result in temporal and spatial expression of proteins and their post-translational modification such as glycosylation. Glycoproteins play important roles in tissue morphogenesis where cell-cell recognition, adhesion and sinaling take part. They participate with other glycoconjugates in the decoration of cell surface, forming the glycocalix that confers an unique molecular identity to the cell lineage. Lectin-based histochemistry has been largely used to demonstrate a wide array of glycan structures that are involved in lineage specification during development. Here we take advantage of nematode *C.elegans*, a well known model organims, to explore the carbohydrate binding properties of plant lectins and unravel the carbohydrate distribution pattern in the whole nematode body. We used two D-Gal binding lectins, jacalin (1) and euphorbin (2), and three D-Man binding lectins, KM+ (3), concanavalin A and lentil lectin. Worms from a mixed population were imobilized on slides previously treated with polylysine. The animals were permeabilized by freeze-cracking and fixed by cold acetone according to Borgonie *et al.*(4). Those slides were then incubated with FITC-labeled lectins. As control of specificity of lectin binding to tissue, FITC-lectins were pre-incubated with haptenic sugars, 0.3 M D-man or D-gal, and all the staining reactions were inhibited. The fluorescence microscopy showed that each lectin provided a distinct staining pattern of *C.elegans* body. Jacalin showed a discontinuous staining pattern from proximal end of the pharynx to the tail. The lectin KM+ presented a difuse staining pattern from the head to the tail through outside of intestine. The lentil lectin stained strongly inside of intestine. Concanavalin A and euphorbin did not cross the cuticule and the possible technical cause is currently being investigated. The different pattern of carbohydrate staining of nematode by these lectins showed that they could

be useful to isolate subpopulation of glycoproteins by affinity chromatography, contributing to shed some light on the glycome of *C.elegans* and the role of glycans during metazoan development. Supported by FAPESP-Brazil (1) Roque-Barreira MC & Campos-Neto A. *J Immunol.,* 134(3):1740-3, 1985 (2) Rosa JC *et al. Glycobiology,* 13 (182-182), 2003 (3) Rosa JC *et al. Protein Sci.,* 8(1):13-24, 1999 (4) Borgonie *et al. Histochemistry,* 100:379-384, 1994

(254) **Domain Organization of the Cst-I sialyltransferase from *Campylobacter jejuni*.**

Michel Gilbert, Marie-France Karwaski, Denis Brochu and Warren W. Wakarchuk

Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa, K1A 0R6..

We have previously reported the cloning of two sialyltransferases (*cst-I* and *cst-II*) from *Campylobacter jejuni* OH4384. The *cst-II* gene was shown to encode a bi-functional (α-2,3/8-) sialyltransferase involved in the sialylation of the lipooligosaccharide. The *cst-I* gene (encoding an α-2,3-sialyltransferase) was cloned from *C. jejuni* OH4384 by activity screening of a plasmid library. However, gene-specific inactivation of *cst-I* (in *C. jejuni* 81-176) showed that it was not essential for lipooligosaccharide sialylation, so the exact role of *cst-I* is still un-defined.The *cst-I* gene encodes a 430 aa protein which appears to be organized in two domains. The N-terminal domain (residues 1-300) shows homology (47% identity) with the bi-functional Cst-II sialyltransferase from the same organism. A series a deletions was performed to determine the minimal Cst-I sequence that retained sialyltransferase activity. Low activity was measured in a construct with residues 1-272 while a construct with residues 1-300 retained almost full activity. The highest activity was observed in the full-length Cst-I (1-430) construct which indicates that the C-terminal domain might have a stabilizing effect. The C-terminal domain (residues 301-430) shows homology with a domain that is present at either the N- or the C- terminal ends of six *C. jejuni* capsule-biosynthesis proteins. Although the C-terminal domain is not essential for enzymatic activity it could haves a role in cell localization.

(255) **Structure of the O-polysaccharide Isolated from Diazotrophic Endophytic Bacterium, *Burkholderia brasiliensis*, Containing an Unusual Branched Sugar, Yersiniose A**

Katherine A. Mattos[1], Adriane R. Todeschini [1], Norton Heise[1], Christopher Jones[2], Jose O. Previato[1] and Lucia MendonÁa-Previato[1]

[1] Instituto de Biof'sica Carlos Chagas Filho, Centro de Ci'ncias da Sa´de, Bloco G,Universidade Federal do Rio de Janeiro, 21944-970, Cidade Universit·ria, Ilha doFund,,o, Rio de Janeiro, RJ, Brasil, [2] . Laboratory for Molecular Structure, NIBSC, Herts EN6 3QG, UK..

Burkholderia brasiliensis is a Gram-negative diazotrophic endophytic bacterium, originally isolated from the interior of roots, stems and leaves of rice in Brazil (Baldani et al.1997, An Acad Bras Ci'n: 69, 116.). An important role in host-plant interaction has been suggested for bacterial lipopolysaccharide (LPS). In the present work the O-specifc polysaccharide chain of *B.brasiliensis* LPS was characterized by compositional analysis, high field nuclear magnetic resonance spectroscopy and mass spectrometry. The O-polysaccharide chain consists of a branched tetrasaccharide repeating unit with the following structure:

→2-α-L-Rha*p*-(1→3)-[α-YerA*p*-(1→2)]-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→

Although C-4-branched 3,6 dideoxyhexopyranose (caryophyllose) have been characterized in *Burkholderia caryophlylli*, our studies show for the first time the presence of a 3,6-dideoxy-4-*C*-(1-hydroxyethyl)-D-*xylo*-hexose (yersiniose A) in *Burkholderia* genus, a sugar previously found as component of LPS from *Yersinia* and *Legionellae* species.

Supported by CNPq, FAPERJ.

(256) **Chemo-enzymatic Synthesis of Protein Conjugates of Schistosome-derived Glycan Antigens and Evaluation of their Immunogenicity in Mice**

A. Kwame Nyame, Ziad S. Kawar and Richard D. Cummings

Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center and the Oklahoma Center for Medical Glycobiology, 975 NE 10th Street, Oklahoma City, OK 73104. USA.

Schistosomes synthesize biantennary N-glycans with LDN (GalNAcβ 1-4GlcNAc) and LDNF (GalNAcβ1-4[Fucα1-3]GlcNAc) epitopes that are immunogenic in infected animals and humans. Antibodies to LDN have been shown to mediate complement dependent cytolysis of juvenile parasites, schistosomula, in vitro. Thus, specific parasite-derived glycans are potential candidates for vaccination and serodiagnosis. We have now generated BSA conjugates with biantennary N-glycans bearing either LDN or LDNF epitopes and have evaluated their immunogenicity in mice to determine their potential as vaccine candidates. N-glycans containing LDN and LDNF were synthesized by remodeling a core biantennary glycopeptide with UDP-GalNAc and recombinant β1,4-N-acetylgalactosaminyltransferase from *C. elegans* and GDP-Fuc and recombinant human α1,3fucosyltransferase IX. The remodeled glycopeptides were dervatized with thiol groups using Traut's reagent and reacted with maleimide activated BSA to form conjugates. Swiss Webster mice were immunized with the BSA conjugates (50 μg) by i.p. route on a 2 wk immunization schedule for a total of 3 immunizations using Freund's or Alhydrogel adjuvants. Control mice were immunized with Freund's or Alhydrogel adjuvants alone. The mice were bled before each immunization and at 2 wk after the last immunization. The sera were analyzed for antibodies by ELISA using biotinylated, remodeled glycopeptides. Mice immunized with either LDN or LDNF conjugates generated both IgM and IgG antibodies in both the Alhydrogel and Freund's adjuvant formulations but the latter adjuvant induced higher antibody responses. The IgG responses commenced after the first boost and was predominantly IgG1 and IgG3 although low levels of IgG2a and IgG2b were also detected. Sera from the control mice lacked any anti-glycan antibodies. This study demonstrates the potential for utilizing synthetic conjugates as vaccine candidates and as antigens for serodiagnosis of schistosomiasis.

(257) **Identification and Partial Characterization of an Active Glucosylceramide Synthase in *Trypanosoma Cruzi*.**

Malena Landoni[1], Vilma G. Duschak[2], Patricia Garavaglia[2] and Alicia S. Couto[1]

[1] CIHIDECAR, Depto de Qu'mica Org·nica, FCEyN, UBA, Argentina, [2] Instituto Nacional de Parasitolog'a, Dr. Mario Fatala ChabÈn, Ministerio de Salud, Argentina..

Biosynthesis of glycosphingolipids involves the sequential action of glycosyltransferases that in mammals seem to be functionally organized in Golgi. However, the key step involves the transfer of glucose from UDP-glucose to ceramide catalyzed by a UDP-Glucose:glucosylceramide transferase (EC 2.4.1.80; glucosylceramide synthase (GCS)) to form glucosylceramide, the precursor of most higher order glycosphingolipids. In the last years, the knowledge of the polypeptide responsable of this enzymatic activity has increased a lot in animals but very little is known about its activity in plants, fungus and bacteria, and nothing in parasitic protozoa. *Trypanosoma cruzi*, the American trypanosome, is responsible for Chagas disease, a serious endemic illness. In epimastigote forms of *T. cruzi*, glycosphingolipid structures have been early detected. On the contrary, very little is known about the enzymes involved in their biosynthesis. In this work we report the presence of an active glucosylceramide transferase (GCS) in epimastigote forms of *Trypanosoma cruzi*, showing a preliminar characterization. The enzyme activity was determined by detection on TLC or HPLC of the NBD-GlcCer formed using NBD-labeled substrates. The enzyme activity was measured in epimastigotes homogenates at different times, concentrations of substrate, total protein amounts and temperatures. The optimal conditions of the reaction were evaluated by quantification of the NBD-glucosylceramide concentration obtained in each case. Two substrates were assayed to examine the specificity of GCS: NBD-Ceramide and NBD-DHCeramide. Interestingly, we have observed that the *T. cruzi* GCS is able to use both substrates but it seems to prefer NBD-DHCeramide in contrast with the mammalian enzyme that has a poor activity with the saturated compound. The efficacy of PPMP (a well-known inhibitor of GCS), tamoxifen, β-conduritol (an inhibitor of glucosylceramidase) and CHAPS on the enzyme activity was also tested. The glycosylation yield resulted only 40% in the presence of PPMP. In *T. cruzi*, tamoxifen increased drastically glucosylceramide synthesis (160 %). Neither the presence of CHAPS nor β-conduritol in the reaction mixture increased the synthesis of GlcCer. In order to get some insight on the subcellular location of this enzyme, we performed subcellular fractionation experiments, by differential centrifugation of epimastigote homogenates. The enriched fractions electrophoresed by SDS-PAGE, and submitted to western blot showed a cross immunoreactive band of 56 kDa with anti-human GCS (a kind gift of Drs.Marks and Pagano) in the microsomal fraction. Glycosphingolipids have demonstrated to play roles in cell growth, survival and in escape from onset of apoptosis. To study their biosynthesis in depth might provide the tools for development of novel chemotherapies for protozoan parasitic diseases.

(258) **Sulfoglycosphingolipids are Biosynthesized by the Intraerythrocytic Stages of *Plasmodium falciparum*.**

Alicia S. Couto[1], Malena Landoni[1], Emilia Kimura[2], Valnice J. Peres[2], Masae Nishioka [3], Hiroshi Nonami[3], Rosa Erra-Balsells[1] and Alejandro M. Katzin[2]

[1] CIHIDECAR, Departamento de Qu'mica Org·nica, PabellÛn II, FCEyN, Universidad de Buenos Aires, Argentina., [2] Departamento de Parasitologia, Inst. de Ci'ncias BiomÈdicas, USP, Brazil, [3] College of Agriculture, Ehime University, Japan. .

Plasmodium falciparum is one of the world¥s most pathogenic microbes, it kills millions annually. Lipid metabolism has been attracting a lot of attention in the last years with respect to basic biology and applications for malaria chemoterapeutic purposes. Recently, we have shown the structural determination of malarial neutral glycosphingolipids. Among acidic glycolipids, gangliosides and sulfoglycolipids are usually found as membrane components, however, previous reports have shown that *P. falciparum* does not biosynthesize sialic acid. In this work we have performed metabolic incorporation of [^{14}C]palmitic acid, [^{14}C]glucose and $Na_2{}^{35}SO_4$ in the three intraerythrocytic stages of *P. falciparum*in order to determine the presence of sulfoglycolipids. Parasites metabolically labeled with [^{14}C]palmitic acid or with [^{14}C]glucose were extracted and fractionated by DEAE-Sephadex column chromatography. The acidic lipids were further subjected to alkaline hydrolysis and analysed. The three intraerythrocytic stages showed the presence of acidic components with both precursors. Moreover, when $Na_2{}^{35}SO_4$ was used as precursor, and the acidic components from the three labeled stages were analysed by TLC, at least two major labeled components SPf1 and SPf2, mainly present in the young trophozoite stage, were detected. Interestingly, both of them showed to migrate below the sulfogalactosylceramide (SM_{4s}), the major sulfoglycolipid detected in uninfected red blood cells. In order to assure the sulfoglycosphingolipid nature of the labeled components [^{14}C]palmitic acid labeled SPF1 was subjected to solvolysis. As expected, solvolysis of SPf1 produced a fast migrating component corresponding to the desulfated product. In addition, when another sample of SPf1 was subjected to methanolysis, a major component coincident with dihidrophingosine was obtained. Analogous results were obtained when the same treatments were performed on SPf2. In order to estimate the percentage of sulfatides among the glycolipids, the [^{14}C]glucose incorporation was used. Sulfoglycolipids are minor components in *P.falciparum*, but interestingly, ring stage and trophozoites biosynthesize twice the amount (20%) of sulfoglycolipids that the schizont stage (10%). In order to make a better characterization of these sulfoglycosphingolipids, an analysis by UV-MALDI-TOF mass spectrometry was performed. Using nor-harmane as matrix in the negative ion mode, it was possible to determine the presence of a d:20 long chain base acylated with hydroxylated fatty acids (C12:0, C14:0). The presence of these sulfated compounds, active participants in adhesion processes in many systems may be related to the crucial role assigned to glycolipids during plasmodial life cycle as well as with their action as potent disrupters of *P. falciparum* erythrocyte rosettes.

(259) **Characterization of Neutral Glycolipids of the Model Filamentous Fungus, *Neurospora crassa*: Altered Expression in Plant Defensin-resistant Mutants**

Chaeho Park[1], Beau Bennion[2], Elizabeth Owuor[2], Isabelle E.J.A. Francois[3], Kathelijne K.A. Ferket[3], Bruno P.A. Cammue[3], Karin Thevissen[3] and Steven B. Levery[2]

[1] The Complex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology, University of Georgia, 220 Riverbend Road, Athens, GA 30602-7229, USA, [2] Department of Chemistry, University of New Hampshire, G229 Parsons Hall, Durham, NH 03824-3598, USA, [3] Centre of Microbial and Plant Genetics, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, B-3001 Heverlee-Leuven, Belgium.

Plants possess an impressive arsenal of antimicrobial compounds which are either constitutively arrayed within certain tissues or synthesized in direct response to attack by pathogens. Among these compounds are defensins, a class of evolutionarily and structurally related small, highly basic, cysteine-rich peptides, many of which display antifungal activity. Defensins are also found in other types of organisms, including insects and humans, as important components of innate immunity. High-affinity binding of plant defensins to fungal cells and membrane fractions has been demonstrated, and also correlated with their antifungal activity (1,2). The structures of human, insect, and plant defensins are consistent with a membranolytic mode of action, but the precise nature of plant defensin interactions with the fungal membrane surface has remained unclear (3). A number of investigations have implicated glycosphingolipids as targets of defensin binding to the fungal membrane (4-6). For example, sensitivity of the yeasts *Pichia pastoris* and *Candida albicans* toward RsAFP2, a defensin isolated from seeds of *Raphanus sativus* (radish) (7), was found to be dependent on *GCS* (6), the gene encoding glucosylceramide synthase (UDP-Glc:ceramide β-glucosyltransferase) (8). In addition, interaction of RsAFP2 was observed with glucosylceramides (GlcCer) isolated from *P. pastoris*, but not from soybean or human (6), which differ in the structures of their ceramide moieties.

The filamentous fungus *Neurospora crassa* has also been used as a system to investigate plant defensin-phytopathogen interactions (1,2,4). Chemically mutagenized *N. crassa* strains selected for resistance to RsAFP2 were found to have dramatically altered glycolipid/sphingolipid expression profiles (4). Although a number of these differences are apparent from even superficial glycolipid profiling by thin layer chromatography (4), the true nature of some alterations, particularly with respect to neutral glycolipid expression, required detailed structural analysis to become apparent. The structural characterization of these neutral glycolipid components, isolated from wild type and defensin-resistant mutant *N. crassa* strains, is the subject of this presentation. Neutral glycolipid components were extracted from wild type and plant defensin-resistant mutant strains of *N. crassa*. These components were purified, and their structures elucidated by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), and tandem MS/CID-MS. Neutral glycosphingolipids in both wild type and mutant strains were characterized as glucocerebrosides (β-glucopyranosylceramide, GlcCer), but those of the mutants were found to possess structurally altered ceramides. In addition, the mutant strains expressed highly elevated levels of a steryl glucoside (GlcSte) identified as ergosterol β-glucoside. The potential implications of these findings with respect to acquisition of defensin resistance in the *N. crassa* mutants will be discussed. References: 1. Thevissen, K., et al (1997) J.Biol.Chem. 272, 32176-32181; 2. Thevissen, K., et al (2000) Mol.Plant Microbe Interact. 13, 54-61; 3. Thevissen, K., et al (2003) Peptides 24, 1705-1712; 4. Ferket, K. K. A., et al (2003) Fungal.Genet.Biol. 40, 176-185; 5. Thevissen, K., et al (2003) FEMS Microbiol.Lett. 226, 169-173; 6. Thevissen, K., et al (2004) J.Biol.Chem. 279, 3900-3905; 7. Terras, F. R., et al (1992) J.Biol.Chem. 267, 15301-15309; 8. Leipelt, M., et al (2001) J.Biol.Chem. 276, 33621-33629.

(260) **Characterization of Lectin-associated Protease Produced by Manila Clams (*Ruditapes philippinarum*) Infected with Perkinsus sp.**

Yoon-Suk Kang[1], SoMi K. Cho[2], Kwang-sik Choi[3], Young-Bae Chung[4] and Moonjae Cho[1]

[1] Department of Medicine, Biochemistry Lab, Cheju National University Medical School , [2] Department of Horticulture and Life Sciences, Cheju National University , [3] School of Applied Marine Science, College of Ocean Science, Cheju National University, [4] Department of Medicine, Parasitology Lab, Cheju National University Medical School .

A specific protease associated with Manila clam lectin (MCL) was purified and characterized from individuals of *Ruditapes philippinarum* that were infected with the protozoan parasite *Perkinsus sp*. This protease did not occur in uninfected clams and was not derived from the parasite itself. Following sample purification, fractions eluted with buffer containing EDTA and N-acetyl-D-galactosamine (galNAc) contained two proteases of different molecular weights. The low molecular weight protease was eluted by galNAc, but there was no proteolytic activity in the PMSF-containing elution buffer. The relative molecular mass of the native protease was determined by Sephacryl S-200HR gel filtration chromatography and found to be 45 kDa. A co-immunoprecipitation assay using an antibody to MCL detected a 45-kDa protease band on a gel following SDS-PAGE. The purified protease was characterized using collagen and bovine serum albumin (BSA). After 2 h or 4 h treatment with the protease, the collagen was degraded but the BSA was not. This indicates that the protease is involved in penetration of connective tissue (matrix) but not in nutrient digestion. The inducible protease associated with MCL might be a major component of innate immunity in the bivalve mollusk *R. philippinarum*

(261) **Identification of Metabolic Precursors of Sialic Acid Biosynthetic Pathway in Plants**

Miti Shah[1], Veer Bhavanandan[2] and Lokesh Joshi[1]

[1] Biodesign Institute at Arizona State University, Tempe, AZ- 85287, [2] College of Medicine, Pennsylvania State University, Hershey, PA-17033.

Sialic acids (SAs) are negatively charged keto sugars that are involved in a wide variety of biological functions. SAs are synthesized by a series of enzymatic reactions involving interconversions and modifications of sugars that include epimerization, phosphorylation and dephosphorylation. The discovery of SAs and sialoglycoconjugates in plants raises important questions about biosynthetic pathways, distribution, metabolism and functions[1]. Researchers have routinely used radioactive precursor sugars to study metabolic pathways and intermediates[2]. To establish and characterize synthesis of sialic acids in plants, cells were grown in the presence of radio-labeled exogenous sugars. The incorporation of labeled sugar precursors into sialic acids provides an insight into the biosynthesis of sialic acids in plants and also indicates similarities to biosynthetic pathway of sialic acid known in other organisms. References: 1. Shah, M., Fujiyama, K., Flynn, R. C., Joshi, L. (2003). Sialylated glycoconjugates in plant cells. Nature Biotechnology 21(12): 1470-1471 2. Varki, A. (1994). Metabolic radiolabeling of glycoconjugates. Methods in Enzymology 230:16-32, Academic Press

(262) **Functional Characterization of the PglH, PglI and PglJ Glycosyltransferases Involved in N-linked Protein Glycosylation in Campylobacter Jejuni**

StÈphane Bernatchez, Jianjun Li, Smita Bhatia, N. Martin Young and Warren W. Wakarchuk
Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa (Ontario), Canada, K1A 0R6.

Protein glycosylation has been found to occur in prokaryotes. It has been extensively studied in the Gram-negative bacterium Campylobacter jejuni in the recent years. Most notably, the N-linked glycan present on more than thirty proteins has been shown to be a heptasaccharide of the sequence GalNAc-a 1,4-GalNAc-a 1,4-[Glcb 1,3-]GalNAc-a 1,4-GalNAc-a 1,4-GalNAc-a 1,3-Bac in which Bac is bacillosamine, a 2,4-diacetamido-2,4,6-trideoxyglucopyranose. Some of the genes of the pgl locus encode the five putative glycosyltransferases that synthesize the Pgl heptasaccharide. A function has been assigned to most of the Pgl enzymes mostly based on the structural examination of the Pgl heptasaccharide following targeted gene inactivation but there is only one case of functional assignment by enzyme characterization. In this work, the glycosyltransferases PglH, PglJ (two putative N-acetylgalactosaminyltransferases) and PglI (putative glucosyltransferase) were studied to define their enzymatic function. The pglH, pglI and pglJ genes were each cloned in the expression vector pCWori+ and expressed in E. coli. The activity of the proteins was investigated in cell lysates using sugar acceptors labelled with a fluorescent tag (FCHASE). Six products were formed in PglH activity assays with UDP-GalNAc and a-GalNAc-FCHASE but no products were formed with UDP-GalNAc and LacNAc-FCHASE. PglI and PglJ were not active when assayed with UDP-Glc (PglI) or UDP-GalNAc (PglJ) with either a-GalNAc- or LacNAc-FCHASE. The three genes were then cloned together and tested for activity in cell lysates using UDP-Glc, UDP-GalNAc and a-GalNAc-FCHASE. The data showed that PglH activity was again present since multiple products were synthesized. PglI activity was also observed and the data suggests that it used one of the products synthesized by PglH as its acceptor. PglI activity was not present in reactions without UDP-Glc, confirming that it is a glucosyltransferase. Carboxy-terminal deletions of PglH and PglJ were engineered to improve protein production and these proteins were also assayed for activity.

(263) **An Endogenous Glycoside, Macrozamin, is Inhibitory Toward a Lectin from Leaves of Japanese Cycad**

Fumio Yagi.
Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima, Japan, 890-0065.

Cycad plants are gymnospermous plants that have been living since the Mesozoic era. Hemagglutination activity is detected in leaves of Japanese cycad (*Cycas revoluta*Thunb.), but not in seeds and roots. In a previous paper (1), we have reported purification and properties of a lectin from leaves of Japanese cycad. Cycad leaf lectin (CRLL) is a member of jacalin-related lectin family, and it has two carbohydrate-recognizing domains. Inhibition of hemagglutination activity of CRLL using trypsinized rabbit erythrocytes showed that 'b'-glycosides as well as 'a'-glycosides are inhibitory. Especially, laminaribiose and Man'b'(1-2)Man were potent inhibitors. On the other hand, cycad plants are known as poisonous plants. Azoxyglycoside with a common aglycone, methylazoxymethanol (MAM), is a toxic principle. Two major azoxyglycosides and several minor neocycasins have been reported. Major ones are cycasin, 'b'-glucoside of MAM, and macrozamin, 'b'-primeveroside ['b'-xylosyl-(1-6)-'b'-glucoside] of MAM. During leaf growth, cycasin amount is in the range of 1-2 mmol/100g leaves, whereas macrozamin temporarily increases and disappears with leaf growth. The maximum amount of macrozamin in leaves of Japanese cycad was 0.5 mmol/100g leaves(5mM). In this study, inhibitory activity of some azoxyglycosides toward hemagglutination activity of CRLL was investigated. Of the azoxyglycosides tested, macrozamin was a strong inhibitor, inhibiting HA of CRLL completely at 3.1 mM. The concentration is lower than the maximum in leaves described above. Cycasin did not show any inhibition at 25mM, and neocycasin A ('b'-laminaribioside of MAM) weakly inhibited at 25mM. These results means that macrozamin, an endogenous glycoside might work as an inhibitor toward CRLL. (1) Yagi *et al*. Eur. J. Biochem., 269, 4335-4341 (2002).

(264) **Biosynthetic Gene Cluster Sequences and Mechanism of Action of E. coli O-antigens**

Wen Yi, Jun Shao, Lizhi Zhu, Mei Li, Yuquan Lu, Steven Lin, Hanfen Li, Kang Ryu, Jie Shen, Hongjie Guo, Qingjia Y. Yao and Peng G. Wang
Departments of Biochemistry and Chemistry, the Ohio State University, Columbus, OH 43210, USA.

The interplay between human blood group antigens (such as ABH blood types) and human natural antibodies (such as human blood group antibodies and anti-α-Gal antibodies) play an important role in transfusion and transplantation. It has been the current understanding that enteric bacteria such as E. coli O86, O127 and O128 are the natural antigens that stimulate the generation of human natural antibodies. Recently, we produced O-antigens from two strains of E. coli O86: O86:K61(B7) and O86:K62:H2, and determined their structures by NMR and methylation analysis. These two strains produced two different structures of O-antigens, and this was further confirmed by our immunological studies with human blood group antibodies and anti-Gal antibodies. Next, we sequenced the O-antigen biosynthetic gene clusters from both strains and surprisingly found out that the two gene clusters are identical. We also realized that the two different O-polysaccharides come from the same O-antigen polymerase Wzy catalyzed polymerization from the same pentasaccharide diphosphate lipid unit. Thus our hypothesis is: There must be other factors that interact with the E. coli O86 O-antigen polymerase Wzy to produce two different polysaccharide products. Our most recent progress on investigating the mechanism of action of O-polysaccharide biosynthesis will be discussed.

(265) **Elucidation of the Biosynthetic Pathway from 5-phosphoribose 1-diphosphate to Decaprenylphosphoryl Arabinofuranose, the Donor of the D-arabinofuranose Residues of Mycobacterial Arabinan**

Dean C. Crick[1], Katar'na Mikuöov·[2], Tetsuya Yagi[3], Marcelle Holsters[4], Wim D'Haeze[4], Michael S. Scherman[1] and Michael R. McNeil[1]
[1] Mycobacterial Reseach Laboratories, Department of Microbiology, Immunology and Pathology, Colorado State University, [2] Department of Biochemistry, Comenius University, Bratislava, [3] Division of Respiratory Medicine, National Center for Geriatrics and Gerontology, Japan, [4] Department of Molecular Genetics, Ghent University, Belgium.

The genus *Mycobacterium* contains species that are pathogenic, such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*, as well as species that are nonpathogenic such as *Mycobacterium smegmatis*. †Although mycobacteria are classified as Gram-positive organisms, they have features of both Gram-positive and Gram-negative bacteria.† The cell envelope of mycobacteria is made up of three major components: a plasma membrane, a covalently linked mycolic acid, arabinogalactan (AG) and peptidoglycan complex, and a polysaccharide-rich capsule-like material.† The major cell wall polysaccharide of mycobacteria is a branched-chain AG in which arabinan chains are attached to the 5 carbon of some of the 6-linked galactofuranose residues.† The arabinan chains are composed exclusively of α-D-arabinofuranose (Ara*f*) residues, most of which are 5-linked.† Branching is introduced at 3,5-linked Ara*f* residues and the non-reducing termini of the arabinan have a [β-D-Ara*f*-(1→2)-α-D-Ara*f*]$_2$-3,5-α-D-Ara*f*-(1→5)-α-D-Ara*f*† motif.

The synthesis of the AG in *M. tuberculosis* begins with the transfer of a GlcNAc-1-phosphate from UDP-GlcNAc to prenyl phosphate followed by an addition of rhamnose (Rha) from dTDP-Rha, thus forming the 'linker region' of the AG.† UDP-galactofuranose (UDP-Gal*f*) is the donor of the gal*f* residues of the galactan.† The Ara*f* residues of the arabinan originate from 5-phosphoribose 1-diphosphate (pRpp) of the pentose phosphate pathway/hexose monophosphate shunt and the immediate precursor of the polymerized Ara*f* is decaprenylphosphoryl-Ara*f*.† All attempts to identify a

nucleotide sugar carrier of Ara*f* have been unsuccessful.† Based on time course, feedback and chemical reduction experiments we propose that decaprenylphosphoryl-Ara*f* is synthesized by the following sequence of events:† 1) pRpp is transferred to a decaprenyl-phosphate molecule to form decaprenylphosphoryl-β-D-ribose 5-phosphate. 2) decaprenylphosphoryl-β-D-ribose 5-phosphate is dephosphorylated to form decaprenylphosphoryl-β-D-ribose.† 3) the hydroxyl group at the 2 position of the ribose is then oxidized to form decaprenylphosphoryl-β-D-2-ketofuranose 4) decaprenyl-phosphoryl-β-D-2-ketofuranose is then reduced to form decaprenylphosphoryl-β-D-Ara*f*. †Thus, the epimerization of the ribosyl residue to an arabinosyl residue occurs at the lipid-linked level and is likely catalyzed by two separate enzymes.

(266) **Identification of a Mannosyltransferase from *Mycobacterium tuberculosis* by Photaffinity Labeling**

Devinder Kaur[1], Marco Macchia[2], Karen M. Dobos[1], John T. Belisle[1], Varalakshmi Vissa[1], Patrick J. Brennan[1] and Dean C. Crick[1]

[1] Mycobacterial Reseach Laboratories, Department of Microbiology, Immunology and Pathology, Colorado State University, [2] Dipartimento di Scienze Farmaceutiche, Universit‡ di Pisa.

Phosphatidylinositol mannosides (PIMs) are precursors of the mycobacterial lipoglycans, lipomannan (LM) and lipoarabinomannan (LAM), which are potent immunomodulators in tuberculosis and leprosy.† The biosynthesis of PIMs, up to PIM_3, is catalyzed by α-mannosyltransferases that utilize GDP-mannose as the sugar donor.† The addition of subsequent mannose (Man) residues to form higher PIMs has been demonstrated to be dependent on polyprenylphosphorylmannose (PPM) as the activated sugar donor.† Recently, several proteins have been identified from *Mycobacterium tuberculosis* that catalyze the formation of glycosidic bonds using GDP-Man as the sugar donor.† However, none of the enzymes which utilize PPM as the sugar donor in mycobacteria have been identified.† Therefore, we undertook a photoaffinity labeling approach to identify this class of enzymes.† We prepared a radiolabeled, photoactive PPM analog that can be used to label proteins that utilize PPM as a substrate for mannosyltransferase reactions.† A cell-free assay was developed that utilized PPM or the photoactive analog as a sugar donor and methylmannoside, methyldimannoside or mannobiose as acceptors.† Exoglycosidase digestion of the products indicated that mannose was added to the acceptor in α1→6 linkage in both cases, demonstrating the utility of the probe as a mannose donor.† Detergent solubilized preparations from *Mycobacterium smegmatis* containing this mannosyltransferase activity were then used as a source of proteins, which were subjected to photolysis in the presence of the photoprobe.† Radiolabeled proteins were separated using two-dimensional electrophoresis, cut from the gel, digested and subjected to HPLC/tandem-MS analysis.† Tandem-MS data was used to identify peptide sequence.† The sequences of these peptides were used to search the *M. smegmatis* genome to identify the labeled proteins.† One of the radiolabeled proteins shared 67% identity with the predicted amino acid sequence of Rv1208 from *M. tuberculosis,* which is annotated as a potential glycosyltransferase.† *Rv1208* was cloned from *M. tuberculosis* H37Rv and expressed in *M. smegmatis* and *Escherichia coli*.† Purified, recombinant protein could be labeled with the photoprobe and cell-free assays containing GDP-[^{14}C]Man and membranes from a *M. smegmatis* strain expressing Rv1208 showed increased synthesis of an alkali-labile, mild acid-stable product with chromatographic properties similar to those of a PIM when compared to control membranes.† Thus, Rv1208 appears to be a mannosyltransferase which is involved in PIM synthesis.

(267) **Lectins in Hemocytes of the Eastern Oyster, *Crassostrea virginica*: Biochemical and Molecular Characterization**

Keiko Saito[1], Satoshi Tasumi[1], Matthew J. Jenny[2], M. Theresa Loreto[1], Gregory W. Warr[2] and Gerardo R. Vasta[1]

[1] Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD, USA, [2] Marine Biomedicine and Environmental Sciences, Medical University of South Carolina, Charleston, SC, USA.

The endoparasitic protozoan *Perkinsus marinus*, causative agent of "Dermo" disease, is responsible massive mortalities in eastern oysters (*Crassostrea virginica*) along the Gulf of Mexico and Atlantic coast. The *P. marinus* trophozoite, its intracellular vegetative life stage, can be found in most host tissues (Perkins 1996), although the mechanism(s) of parasite infection and disease transmission is not fully understood. It has been proposed that it occurs mainly trough filter-fed parasites that are phagocytosed by hemocytes, where they survive and proliferate. Lectins either present in host plasma or those associated with hemocytes may play a role in parasite entry, by opsonizing *P. marinus* infective forms and enhancing their entry into the host hemocytes. Thus, although no strict host specificity has been demonstrated for *Perkinsus* spp., such host-parasite recognition mechanism would further explain the association of *Perkinsus* spp. with selected mollusk species: *P. marinus* and *P. andrewsi* in oysters; *P. olseni* in abalone; *P. atlanticus*, *P. cheasapeaki*, *P. andrewsi* in clams; and *P. qugwadi* in scallops. The presence of galactose-binding lectins in oyster plasma and hemocytes has been previously determined with biochemical approaches (Vasta *et al.*, 1986). Sequences of 3 C-type lectins and a C1q-like protein were identified by BLAST analysis of a *C. virginica* hemocyte EST library. Clones of interest (CvEST013, 074, 085 and 496) were resequenced, and the full ORFs obtained. CvEST013 and 496 were 84% identical (about 180 amino acids, 27 kDa), to a putative galactose-binding lectin from the sea urchin. CvEST085 is similar to a mannose-binding C-type lectin (193 amino acids, 28 kDa) with the EPN motif at the C-terminal end. Using sugar affinity chromatography (Lac-, GlcNAc-, Man- and Fuc-Shepharose), several lectins were isolated from *C. virginica* hemocyte extracts and cell-free hemolymph. On SDS-PAGE, multiple bands were identified in each sugar-bound fraction, suggesting a complex lectin repertory. Preliminary lectin-blot and histochemical studies with clonal *Perkinsus* spp. suggest a differential distribution of galactosyl residues on the cell surface of various *Perkinsus* spp. We hypothesize that galactose-binding lectins play a role on the selective recognition, opsonization and phagocytosis of *Perkinsus* spp. by *C. virginica* hemocytes. [Supported by NOAA/MD Sea Grant: SA7528068-H to G. R. V.]

(268) **Properties of Glycan Oligomers from Developing Cotton Fibers**

Allen K. Murray[1], Robert L. Nichols[2], Robert J. Seward[3] and Catherine E. Costello[3]

[1] Glycozyme, Inc., 17935 Sky Park Circle, Ste. E, Irvine, CA 92614 , [2] Cotton Incorporated, 6399 Weston Parkway, Cary, NC 27513, [3] Mass Spectrometry Resource, Boston University School of Medicine, Boston, MA 02118.

Cotton fibers are single cells arising from the exterior coat of the cotton seed. The cell walls of mature cotton fibers are primarily glucans, containing greater than 95% cellulose, but exhibit complex three dimensional structure, comprised of a primary cell wall and concentric, diurnally deposited secondary cell wall elements. The oligomers may be extracted directly from the fibers with 0.1N HCl at 100∞ or by the same method from a particulate precipitate released by sonication of the fibers in cold water. The oligomers are more abundant at 21 days post anthesis (DPA), when primary cell wall biosynthesis is nearing completion, and seconday cell wall synthesis is rapidly increasing, than at 35 or 45 DPA or from mature fibers. Previous research indicates that the content of the oligomers varies in response to developmental (DPA), physiological (time of day) and environmental (stress) variables. Structural studies on the oligomers are on-going. The solubility of the oligomers has been investigated to optimize their extraction and to increase our understanding of conditions required for the conversion of cell wall precursors from the soluble to the insoluble state. Complete extraction is essential to investigate quantitative relationships between the oligomers and the developed fibers. Following extraction the oligomers were chromatographed on molecular sieve columns, either BioGel P-4 or Sephadex G-25. Each fraction collected from the columns could then be resolved into10-12 peaks by high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The UV-MALDI mass spectra were obtained for each of the fractions. The dominant ion series in each case was that of (hexose)n up to n=32. Treatment of the oligomers with a highly purified endo-fl-1,4-glucanase (Megazyme) readily converts the oligomers with longer retention times to those with shorter retention times and liberates predominantly glucose. The oligomers also contain galactose and mannose. As the size of the oligomers increase, the relative amounts of galactose and mannose appear to be approximately constant, with the difference in composition between the oligomers being an increase in the amount of glucose. We have hypothesized that the oligomers play a role in the biosynthesis of the cell wall of developing cotton fibers. Murray, A.K., R. L. Nichols, and G. F. Sassenrath-Cole, 2001, Phytochemistry, 57(6):975-986.

(269) **A Predicted Diglycosyltransferase Gene Contributes to Spore Coat Polysaccharide Biosynthesis in *Dictyostelium discoideum***

Phuong Nguyen, Hanke van der Wel, Talibah Metcalf and Christopher M. West

Dept. of Biochemistry & Molecular Biology, Oklahoma Center for Medical

Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104 USA.

The spore coat is a trilaminar cell wall that is formed de novo at the cell surface of differentiating spores of Dictyostelium. It is assembled from a vesicular pool of stored protein and a Gal/GalNAc-rich polysaccharide (GPS), a late-synthesized pool of protein, and cellulose. The GPS is recognized by the lectins RCA-I and soybean agglutinin and is localized to the inner and middle layers of the coat, but its significance for spore coat structure and function is not known. In a related species, Dictyostelium mucoroides, a similar spore polysaccharide consisting of the repeating trisaccharide Galβ1,3(Galβ1,6)GalNAcα1,3- has been described (Sakurai et al., Comp. Biochem. Physiol. Part B 132:541-549, 2002). A search of the genome sequence of D. discoideum for potential Golgi-localized two-domain diglycosyltransferase genes, which might direct formation of the repeating disaccharide of the backbone of its GPS, yielded two candidates (gtdA and gtdB). Disruption of gtdA by homologous recombination abolished labeling of spores with the RCA-I lectin. Spores produced by gtdA-mutant spores were less resistant to hypertonic stress, less hydrophobic, and exhibited increased permeability to a macromolecular dye. Co-development of mutant and wild-type cells indicated that incorporation of GPS into coats is cell autonomous. The predicted glycosyltransferase domains of gtdA appear to belong to families GT-2 and GT-4 (Coutinho et al., J. Mol. Biol. 328:307-317, 2003). gtdA encodes a predicted N-terminal signal anchor sequence, but family GT-2 and GT-4 sequences are typically within or oriented toward the cytoplasm if membrane associated. Since the GPS is stored in prespore vesicles prior to secretion during spore coat assembly, further studies are required to determine the site and topology of GtdA function, and to determine its enzymatic contribution to polysaccharide formation. (Supported by NSF MCB-0240634 & NIH R01-GM-37539)

(270) **Glycosylated Aeruginosins, Non-ribosomal Peptides from Cyanobacteria: Structure Elucidation and Isolation of the Corresponding Aeruginosin Synthetase Gene Cluster**

Guntram Christiansen[1], Keishi Ishida[2], Wesley Yoshida[1], Rainer Kurmayer[3], Elke Dittmann[2], Thomas Boerner[2] and Thomas Hemscheidt[1]

[1] University of Hawaii, Dept. of Chemistry, 2454 McCarthy Mall, Honolulu, HI 96822, USA, [2] Humboldt University, Institute for Biology, Chausseestr.117, 10115 Berlin, Germany, [3] Institute for Limnology, Austrian Academy of Sciences, Mondseestr. 12, 4972 Mondsee, Austria.

Cyanobacteria are known to be a rich source of bioactive secondary metabolites. A large number of these bioactive compounds are peptides. Due to their extraordinary structural features the biosynthesis of these peptides cannot be explained by the ribosomal pathway. Instead, these compounds are synthesized non-ribosomally by a pathway known as the thio-template mechanism. This mechanism of biosynthesis is so far exclusively found in prokaryotes and lower eukaryots. Up to date more than 300 different amino acids and derivatives have been shown to be part of peptides synthesized by non-ribosomal peptide synthetases (NRPS). Beside this enormous diversity of the amino acid residues, the peptides can be further modified by specialized enzymes. These modifications which include N-, O- and C-methylations, halogenations, and glycosylations further enhance the extraordinary structural diversity found in non-ribosomal (NR)synthesized peptides. While glycosylated NR peptides are prevalent in Streptomyces, Actinomyces, Bacillus and fungi, there are so far only few reports of glycosylated peptides occuring in cyanobacteria. Aeruginosins, potent thrombin/trypsin inhibitors isolated from different cyanobacterial genera, are linear peptides consisting of Hpla (p-hydroxyphenyllactic acid), D-Leu, Choi (2-carboxy-6-hydroxyoctahydroindole) sulfate and agmatine. Glycosylated variants of aeruginosins were so far only detected in the genera Anabaena and Planktothrix. We elucidated the structural variants produced by the strain Planktothrix agardhii NIVA/CYA 126/8. Interestingly the aeruginosins of this strain can be glycosylated at two positions, either at the D-hydroxy-Leu or at the Choi residue. We attribute the biosynthesis of these two variants to a relaxed substrate specificity of a proposed glycosyltransferase. Recently we isolated a gene fragment which was used in an insertional gene inactivation experiment. Analysis of transformants revealed that aeruginosins are missing in the mutants, indicating that the inactivated gene is part of the responsible biosynthetic gene cluster in Planktothrix agardhii CYA/NIVA 126/8. The ongoing sequencing of the entire aeruginosin gene cluster revealed an ORF with high homology to glycosyltransferase genes. We report the isolation and structure elucidation of two new glycosylated aeruginosins and the insertional inactivation of the aeruginosin gene cluster in the strain Planktothrix agardhii NIVA/CYA126/8.

(271) **Identification of Aminosugar-containing Glycoproteins in Bacteria Using Unnatural Azidosugar Anabolism**

Brian L. Carlson[1], Scott T Laughlin[2] and Carolyn R. Bertozzi[1,2,3]

[1] Departments of Molecular & Cell Biology, [2] and Chemistry, University of California, Berkeley, [3] Howard Hughes Medical Institute.

Glycoproteins are well-known mediators of cell-cell interactions in multicellular eukaryotic organisms. Like their eukaryotic counterparts, several bacterial glycoproteins have also been found to mediate cell-cell interactions (1, 2). Nevertheless, the spectrum of known bacterial glycoproteins is limited, in part, due to a lack of effective analytical techniques. Considering the potential role of these glycoproteins in mediating pathogen-host interactions, and the ever-increasing need of new antimicrobial therapeutics designed to interfere with these interactions, new tools to discover glycosylated proteins are needed. Recently, our lab has developed a method for glycoprotein detection by exploiting the anabolism of unnatural azide-containing sugars (3). Here we report the progress towards using this new technique to detect and identify novel bacterial glycoproteins using E. coli and mycobacteria as model organisms.

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3. Saxon E, Luchansky SJ, Hang HC, Yu C, Lee SC, Bertozzi CR. J Am Chem Soc. 2002, 124, 14893-14902.

(272) **Expression of *Dolichos biflorus* LNP in *Lotus japonicus* Broadens its Host-Strain Specificity.**

Gurpreet Kalsi[1], Nicholas J. Roberts[1,2], R. Bradley Day, Gary Stacey[3] and Marilynn E. Etzler[1]

[1] Section of Molecular and Cellular Biology, University of California, Davis, CA95616, USA, [2] Present address: Plant Breeding & Genomics, AgResearch Grassland Research Centre, Private Bag11008, Tennent Drive, Palmerston North, New Zealand, [3] Department of Plant Microbiology and Pathology, University of Missouri-Columbia Columbia, MO 65211, USA..

The initiation of the nitrogen-fixing symbiosis between rhizobia and legumes is host- strain specific. This specificity is determined by the type of lipochitooligosaccharide Nod factor signal produced by a particular rhizobial strain and the ability of a leguminous species to recognize that signal. Previous work in our laboratory identified a Nod factor-binding protein, called LNP, that is present on the root hair surface in the zone of nodulation, the site at which rhizobia bind to the roots. A variety of experiments conducted by our laboratory strongly suggest that LNP may function in the initiation of the rhizobial-legume symbiosis. In an effort to further evaluate the role of LNP in this process, we transformed the legume *Lotus japonicus* with LNP (Db-LNP) from the legume *Dolichos biflorus*. We have identified two independent lines which express Db- LNP in young *Lotus japonicus* roots. A combination of immunoblots, in situ hybridization and immunolocalization studies show that Db-LNP is expressed in young roots of *Lotus japonicus* and that this protein is present on the surface of young root hairs in the zone of nodulation. These transformed *Lotus japonicus* lines form typical 'Shepherd's crook' structures and nodule like structures in response to *B. japonicum* USDA110 inoculation, a symbiont of *Dolichos biflorus*, thus broadening the host-strain specificity of *Lotus japonicus*. *Lotus japonicus* lines transformed with *Dolichos biflorus* seed lectin did not show these responses. To further test whether the interaction between Db-LNP expressing *Lotus japonicus* lines and *B. japonicum* USDA110 is mediated through the specific Nod factors, plants were also inoculated with Nod factor deficient mutant strains of *B. japonicum* USDA110. No nodule like structures and 'Shepherd's crooks' were seen in response to these mutant strains. These results further support our hypothesis that Db-LNP may serve as a Nod factor receptor and play a role in the initiation of rhizobium-legume symbiosis. (This research was supported by NIH Grant GM21882 and by a grant from Ceres, Inc.)

(273) **Intra-peritoneal Macrophage is Activated by Uptake of Mannose Conjugated Liposome**

Yuzuru Ikehara[1], Naoya Kojima[2], Hayao Nakanishi[1], Touru Yoshii[2], Le

Biao[2], Toru Niwa[1,3] and Masae Tatematsu[1]
[1] Division of Oncological Pathology, Aichi Cancer Center Research Institute, Nagoya 464-8681 JAPAN, [2] Department of Applied Biochemistry Tokai Univ. Hiratsuka 259-1292 Japan, [3] 2nd Department of Internal medicine, Wakayama Medical Univ. Wakayama 641-0086 Japan.

[Background] Macrophage mannose receptor (MMR) is one of mannose binding proteins, which is preferentially expressed on macrophage cell surface. MMR has a large extracellular domain comprising an a cystein-rich, a fibronectin type II, and eight C-type lectin domain, and a short cytoplasimic tail that carries both conserved tyrosine-based and dihydrophobic based putative endocytosis motif. Though the receptor function is considered to respectively link with mannose binding and ingestion, it has not been known whether the endocytosis starts upon MMR binding to Mannose residues. [Methods] In order to explore the contribution of mannose binding on macrophage activation, uptake efficiency was investigated in peritoneal macrophage using either mannotriose (Man3) conjugated liposome (MCL) or bare (without Man3) liposome containing fluorescein labeled BSA. MCL was prepared from phosphatidylcholine, cholesterol and a neoglycolipid consisting of Man3 and dipalmitoyl-phosphatidylethanolamine, while bare liposome lack the neoglycolipid. Uptake efficiency of MCL or bare liposome by macrophage was detected by FACS analysis, and the endocytosis was confirmed by detection of fluorescein signals in the cytoplasm using confocal microscope. Macrophage recruitment (or the accumulated foci in peritoneal cavity) was visualized with the fluorescein dissection microscope. [Result] One hour after MCL injection into peritoneal cavity of BALB/c mice, fluorescein signal was detected in more than 70 % of intra-peritoneal mononuclear cells that exhibited F4/80 macrophage marker. On the contrast, injected bare liposome containing fluorescein labeled BSA was not exclusively detected (less than 6 %) in F4/80 positive cells. J774A cells express MMR on the surface, whose tyrosine phosphorylation was increased upon MCL uptake, suggesting that macrophage is activated by uptake MCL through the mannose receptor. To further evaluate roles of ingestion of MCL on the activation, we traced macrophage with the fluorecsein signal to know where the cells were recruited. Six hour after injection of MCL, macrophages accumulation could be detected at extra nodal lymphoid tissues in omentum, so called milky spot. Maximal accumulation was seen around 24 hour after injection. No apparent signals were observed when bare liposome was injected. [Conclusion] Taken together, these results suggest that residential macrophage of peritoneal cavity is activated on uptake liposome through the Man3 recognition, resulting in recruitment to milky spot that is extra nodal regional lymphoid tissue. Supported by Grant-in-Aids for Scientific Research on Priority Area from Ministry of Education, Culture, Sports, Science and Technology of Japan (YI;16790196), and in part by a grant for Hi-Tech Research from Tokai University and a grant for AIDS research from the Ministry of Health, Labor, and Welfare of Japan(NK).

(274) Modification of FGF-1, Preferentially with Heparan Sulfate but Not Chondroitin Sulfate

Masahiro ASADA, Emi HONDA and Toru IMAMURA
AIST, Tsukuba Central #6, 1-1-1 Higashi, Tsukuba, Ibaraki, 305-8566 Japan.

We previously reported that augmented activity of PG-FGF-1, a chimeric protein of FGF (fibroblast growth factor)-1 with ectodomain of syndecan-4, was ascribed to heparan sulfate (HS) sugar chain(s) that modifies this protein. As the utilized domain of syndecan-4 contains three glycosaminoglycan (GAG) attachment sites, the PG-FGF-1 was modified with both HS and chondroitin sulfate (CS). Here, in an attempt to obtain a FGF-1 chimeric protein with single HS modification, we created a short form of PG-FGF-1 (SPG-FGF-1) that contains only one GAG attachment site.

A cDNA encoding a secretion signal and one GAG attachment site of syndecan-4 (48 amino acids) fused to the N-terminus of FGF-1 was transfected to COS-1 cells in an expression vector. The secreted protein was resolved by SDS-PAGE and analyzed by immunoblotting after digestion with GAG degrading enzymes. We found that the SPG-FGF-1was dominantly modified with HS, while PG-FGF-1 was modified with both HS and CS. Furthermore, the SPG-FGF-1 was superior to PG-FGF-1 in its mitogenic activity towards FGF-receptor expressing Ba/F3 cells in the absence of heparin.

Thus the SPG-FGF-1 is superior to PG-FGF-1 in its easiness of quality control and usefulness as a therapeutic agent. The SPG-FGF-1 also provides information of a peptide sequence that is preferentially modified with HS.

(275) Galactose Sulfate as a Structural Relative of Tyrosine Phosphate.

Clifford A. Lingwood[1,2], Murugesapillai Mylvaganam[1], Farah Minhas[1], Beth Binnington[1] and Regis Pomes[1,2]
[1] Research Institute, Hospital for Sick Children, Toronto, Ontario, M4G 1X8, Canada, [2] Departments of Biochemistry and Laboratory Medicine& Pathobiology.

Cell surface 3-sulfogalactosyl ceramide (sulfatide, SGC) provides glycosphingolipid receptors for a variety of ligands involved in cell adhesion and microbial pathology. Tyrosine phosphate(PY) provides the basis of many recognition motifs in intracellular signalling cascades. Based on the recognition of both SGC and tyrosine sulfate by several extracellular ligands (selectins, HIV gp120, von Willebrand factor VIII), we have considered the relative receptor function of SGC and PY. From the binding of Mab anti-SGC, hsp70 and anti phosphotyrosine antibodies to various SGC and PY analogues, we show that the sulfogalactose moiety shares recognition elements in common with tyrosine phosphate. We demonstrate examples where ligand binding is retained following phosphate/sulfate substitution in SGC, tyrosine/galactose substitution in SGC and sulfate/phosphate substitution in PY. Tyrosine sulfate, when coupled to different (lipid) isoforms of phosphatidyl ethanolamine, shows remarkable lipid-dependent modulation of ligand binding activity, similar to the lipid-dependent modulation of glycolipid receptor function we, and others, have reported in many studies, suggesting a similar microenvironmental modulation of tyrosine sulfate receptor function. We propose a continuous spectrum of recognition epitopes from tyrosine phosphate through to galactose sulfate, both in terms of the charge and ring substitutions. Analysis of the substrate bound cocrystal of arylsulfatase A, an esterase which cleaves both SGC and PY, provides a structurally defined system which further supports this model. The biological implications of the proposed receptor cross-reactivity are significant.

(276) Concentration of Carbohydrates in Lipid Raft Fraction of Rat Cerebellum

Kohji Kasahara[1,2], Naoko Suzuki[1,2], Kohei Yuyama[1,2] and Yutaka Sanai[1]
[1] Tokyo Metropol. Inst. Med. Sci., [2] PRESTO, JST.

Lipid rafts are ganglioside-rich microdomains of the plasma membrane, and are implicated in signal transduction because a variety of signaling molecules are associated with them. To clarify raft-mediated signal transduction, we have isolated ganglioside binding proteins from rat cerebellum by co-immunoprecipitation with anti-ganglioside antibody. Previously we demonstrated that anti-ganglioside GD3 antibody (R24) co-immunoprecipitated 40, 53, 56 and 80 kDa phosphoproteins and 135kDa protein. 40 kDa protein was identified as α subunit of trimeric G protein, Go. 53/56 kDa proteins were identified as src-family tyrosine kinase Lyn. 80 kDa protein was identified as Csk-binding protein Cbp, a substrate for src-family kinase. 135kDa protein was identified as GPI-anchored neuronal cell adhesion molecule TAG-1, N-linked glycoprotein. These proteins and ganglioside GD3 were present in lipid raft fraction on sucrose density gradient. Furthermore, phosphacan, a high affinity ligand of TAG-1, is chondroitin sulfate proteoglycan. Phosphacan transduces signal via TAG-1/Lyn in lipid rafts. These observations suggest that various glycoconjugates such as gangliosides, GPI-anchors, N-glycans and proteoglycan are concentrated in outer leaflet of lipid rafts. Therefore, we investigated the presence of carbohydrates in lipid raft fractions of rat cerebellum. Coomassie Brilliant Blue staining demonstrated most cellular proteins were present in non-raft fraction and less than 1% of proteins was detected in lipid raft fraction. In comparison with proteins, a considerable amount of carbohydrates were detected in lipid raft fractions by periodic acid-Schiff staining. Con A and ABA lectin staining also demonstrated that a considerable amount of carbohydrates were detected in lipid raft fractions. Furthermore, a considerable amount of HNK-1 epitopes were detected in lipid raft fraction by immunoblotting. These observations suggest that carbohydrates are concentrated in lipid rafts of rat cerebellum.

(277) AFM Observation of GM3-enriched Microdomain Composed of Lipids Extracted from Mouse B16 Melanoma Cells

Kazutoshi Iijima, Teruhiko Matsubara and Toshinori Sato
Department of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan

The glycosphingolipids are enriched in lipid domain called 'raft' composed of sphingolipids and cholesterol. The glycosphingolipids in cell membrane play physiochemical and biological roles by self-assembling. To obtain information of distribution of ganglioside GM1 in phospholipids, an atomic

force microscope (AFM) has been employed in our previous study. It was shown that GM1 formed small domain of submicrometer scale in various phosphatidylcholine and other lipids. In this study, the assembled structure of ganglioside GM3 (Neu5Acα2-3Galβ1-4Glcβ1-1'Cer) in model bilayers composed of lipids extracted from mouse B16 melanoma cells was investigated by an AFM. B16 cells overexpress GM3 as ganglioside. AFM measurements indicated that the lipids from B16 cells formed two kinds of domain having different heights, the highest domain was recognized by wheat germ agglutinin (WGA). Therefore the highest domain was considered to be a GM3-enriched microdomain. To know the mechanism for the formation of GM3-enriched microdomain, the surface topology of bilayer composed of three or four kinds of chemically identified lipids was studied. The lipid mixture of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC)/sphingomyelin (SM)/cholesterol (50:10:35 by mol) formed the domain composed of SM and cholesterol. The area of the domain was quantified from the histogram of height distribution using image analysis software. The area of the domain was 16.5%, which is corresponding to the molecular area of SM containing cholesterol uniformly distributed in POPC and SM. The area of lower phase was 83.5%, which is corresponding to the molecular area of POPC containing cholesterol. By the addition of 5 mol% GM3 in POPC/SM/cholesterol (50:10:35) mixed membrane, the AFM images was almost similar to that of the lipid extracts from B16 cells and showed the existence two kinds of domain. The area of two domain having different height was 18.4% and 7.0%, which is correspond to the molecular area of SM and GM3, respectively. Cholesterol is considered to uniformly distribute in POPC, SM and GM3. The domain of GM3/cholesterol was observed only in the domain of SM/cholesterol, and the domain was recognized by WGA. The topological image and WGA-binding affinity of GM3/POPC/SM/cholesterol mixed lipid membrane were good agreement with those of lipid extracts from B16 cells. The present results strongly supported that GM3 have a potential to form a microdomain in lipid membrane. We measured the AFM images of membrane composed of lipids extracted from animal cells. Since lipid raft has high mobility, it had been considered that glycolipids are uniformly distributed in lipid raft (F. Galbiati et al. Cell 2001, 106, 403-411). However we showed that the glycolipids was self-assembled in the domain composed of SM and cholesterol. Our present study also might support the previous report that glycosphingolipids are not essential in raft formation (G. A. Ostermeyer et al. J. Biol. Chem., 1999, 274, 34459-66). The AFM observation for the topology of lipid membrane and the lectin-binding to lipid domain is very useful to know the structure and the recognition function of the glycolipid-enriched microdomain.

(278) **Novel Functional Role of GPI-anchor Glycan in IL-18 Induced Signaling**

Keiko Fukushima[1], Masayuki Izumi[2], Hironobu Hashimoto[2] and Katsuko Yamashita[1]

[1] Dept. of Biochemistry, Sasaki Institute, [2] Department of Life Science, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology.

Interleukin-18 (IL-18) induces T cells and natural killer cells to produce not only interferon-γ (IFN-γ) production but also other cytokines by binding to the IL-18 receptor α and β subunits. However, little is known about how IL-18, IL-18Rα, and IL-18Rβ form a high-affinity complex on the cell surface and transduce the signal. First, we found that IL-18 specifically binds to GPI-anchor glycan using the plate coated with human alkaline phosphatase (hAP). To investigate whether the carbohydrate recognition ability of IL-18 is involved in the mechanism of IL-18 function, the haptenic effects were examined using human leukemia KG-1 cells, which produce IFN-γ by stimulation of IL-18. Interestingly, in the presence of mannose 6-phosphate, IL-18 partially inhibited IFN-γ production of KG-1 cells, on the contrast, dimannose diphosphate enhanced IFN-γ production. Moreover, dimannose diphosphate also enhanced tyrosine phosphorylation of KG-1 cells by IL-18 stimulation, suggesting that GPI anchor glycan modulates IL-18 function. Secondly, we found that IL-18 exclusively recognizes the third mannose 6-phosphate diester and that IL-18Rα recognizes the second mannose 6-phosphate of GPI anchor glycan by using the plate method and SPR method. Since a β-N-acetylglucosamine residue of the second GlcNAcβ1-phosphate-6mannose residue must be removed to be recognized by IL-18Rα, the β-N-acetylglucosamine residue that protects the exposure of the second mannose 6-phosphate diester in the GPI-anchor glycan may regulate the immune response to IL-18 via toll-like receptors. This is the first report showing that a GPI-anchor glycan binding to both IL-18 and IL-18Rα triggers the IL-18 delivered signaling.

(279) **Specific Cell Adhesion via Cell Membrane Glycosyl Transferase onto Glycopolymers**

Kenichi Hatanaka, Maria Carmelita Kasuya and Kunihiko Iwamoto

Institute of Industrial Science, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan.

Specific adhesions of several kinds of cells onto glycopolymers by using cell membrane galactosyl transferase were investigated. 4 kinds of polystyrene having D-glucose, D-galactose, N-acetyl-D-glucosamine, and uridine were synthesized. 3T3-L1 was well adhesive on the surface of uridine-containing polystyrene, while it was not adhesive on the glucose-, galactose-, or N-acetyl-D-glucosamine-containing polymer. However, in the presence of UDP, 3T3-L1 adhesion on the surface of glucose-, galactose-, or N-acetyl-D-glucosamine-containing polymer was obseved. By considering that the affinity of galactosyl transferase to N-acetyl-D-glucosamine increases in the presence of UDP, galactosyl transferase-mediated cell adhesion may occur. 3T3-NIH, B-16, and PC-12, which had the galactosyl transferase at cell surface, were also adhesive on the uridine-containing polymer. On the other hand, cell membrane galactosyl transferase negative cells such as HeLa were not adhesive on the glycopolymers. On the glucose-containing polystyrene, specific adhesion of 3T3-L1 occurred in the presence of α-lactalbumin as well as UDP. However, the α-lactalbumin was not effective on the cell adhesion on the N-acetyl-D-glucosamine-containing polymer. There were the optimum concentrations of UDP and α-lactalbumin for the specific cell adhesion on the glucose- and N-acetyl-D-glucosamine-containing polymers. Specific control of cell adhesion via cell membrane galactosyl transferase by using 4 kinds of ligand, which are glucose, galactose, N-acetyl-D-glucosamine, and uridine, and 2 kinds of effective molecule of UDP and α-lactalbumin is also investigated.

(280) **Role of Lysosomal Glycosidases and Activator Proteins in Lipid Antigen Presentation by CD1 to T lymphocytes**

Dapeng Zhou.

Department of Pathology, University of Chicago, 5841 S. Maryland Ave, MC1089, Chicago, IL60637.

Pioneering investigations of the molecular basis of lysosomal storage diseases have led to a series of discoveries on glycosidases that metabolize the complex glycoconjugates, their activator proteins, and the trafficking mechanisms for both the enzymes and their substrates. We have used genetic approaches including gene targeted mice and RNAi silenced cell lines to dissect the pathways involved in the trafficking, processing and loading of glycolipids onto CD1, an MHC-like glycoprotein family specialized in microbial and self lipid antigen presentation. We found that the development and the function of CD1/lipid specific T cells are critically regulated by enzymes and activator proteins of glycolipid metabolism. In addition, we have used cell-free assays to elucidate the biochemical basis of their function in the context of CD1-mediated lipid antigen presentation. Because the process of immune recognition of lipid antigens by T cells of the immune system appears to have extensively coopted the existing machinery for glycolipid metabolism, we propose various T cell assays of unprecedented sensitivity and specificity to monitor and quantitate glycolipid metabolism in vivo. These new assays might be useful for diagnosis, prognosis and treatment of lipid storage diseases.

(281) **Characterization of Galactosylceramide Expression Factor-1 in MDCK cells and C3H10T1/2 cells**

Kiyoshi Ogura, Yuuko S. Niino and Tadashi Tai

Department of Tumor Immunology, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan.

We previously reported that galactosylceramide expression factor-1 (GEF-1), a rat homologue of hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs/Hgs), induces galactosylceramide and/or sulfatide expression and morphological changes in epithelial cells (1, 2, 3). Here we show that GEF-1 induces myogenesis in MDCK and C3H10T1/2 cells. GEF-1 overexpression in MDCK cells (MDCK/GEF-1) appeared to promote trans-differentiation to myoblasts that express MyoD and myosin heavy chain. MDCK/GEF-1 cells also expressed DNA-binding activities of transcription factors (MyoD, E47 and MEF-2) that are essential for myogenesis. These findings suggest that GEF-1 induces MDCK cells to trans-differentiate into an early stage of myogenesis. Subsequently, we tested whether GEF-1 could induce myogenesis in C3H10T1/2 mouse fibroblasts, which have the

potential to differentiate into myoblast-like cells. GEF-1 overexpression in C3H10T1/2 cells (10T/GEF-1) appeared to promote differentiation to myoblasts morphologically. Indeed, 10T/GEF-1 cells expressed MyoD and myosin heavy chain, and DNA-binding activities of transcription factors (MyoD, E47 and MEF-2) that are essential for myogenesis. Taking these results together, it is suggested that GEF-1 have the potential to induce myogenesis (4). 1. Ogura K, Kohno K, Tai T. *J. Neurochem* . 1998; 71: 1827-36., 2. Ogura K, Tai T. *Glycobiology*. 2001; 11: 751-8., 3. Ogura K, Tai T. *Neurochem. Res*. 2002; 27: 779-84., 4. Ogura K, Niino YS, Tai T. *Arch. Biochem. Biophys*. 2004; 426: 279-85.

(282) **Glycosphingolipid Receptor for Fish Pathogen, *Vibrio trachuri***

Shinichi Chisada[1], Yasuhiro Horibata[1], Masanori Inagaki[2], Yoichiro Hama[3], Nozomu Okino[1] and Makoto Ito[1]

[1] Department of Bioscience and Biotechnology, and, [2] Faculty of Pharmaceutical Science, Graduate School Kyusyu University, Fukuoka, Japan, [3] Faculty of Agriculture, Saga University, Saga, Japan.

Accumulating evidence indicates that in mammals many pathogenic bacteria initially bind to glycosphingolipids, and thereafter penetrate into host cells. It has also been reported that many intestinal bacteria are adherent to glycosphingolipids, and subsequently, are firmly fixed to the host cell surface as symbionts. However, in non-mammalian systems, the molecular mechanism of the interaction between bacteria and host cells/tissues is little understood.Å@Clarification of the molecular mechanism of the interaction between the fish intestine and bacteria should provide insight for defense against the invasion of pathogens and development of antibiotics and probiotics. Here, we report that a fish pathogen, *Vibrio trachuri*, binds to GM4 ganglioside isolated from the intestine of red sea bream, *Pagrus major* (Japanese name, Madai). First, total lipids, extracted from the fish intestine by chloroform/methanol (2/1, v/v), were subjected to Folch's partition and then the lower phase was purified with a Sep-Pack Silica cartridge after treatment of the sample with 0.2 M KOH. The GSL fraction was applied to a TLC plate which was developed with chloroform/methanol/0.02% $CaCl_2$ (5/4/1, v/v). Three major GSLs (tentatively designated IGL-1, 2, and 3) were detected when visualized by spraying with orcinol-H_2SO_4 reagent. Second, we isolated bacteria from the intestine of red sea bream and performed a binding assay using IGLs and isolated bacteria which had been labeled with ^{35}S-methionine. Among the 100 isolates tested, 20 strains were able to bind IGL-2. The bacterium which exhibited the strongest binding activity to IGL-2 was identified as *Vibrio trachuri*, a well known fish pathogen, by conventional and 16S rDNA analyses. IGL-2 was identified as GM4 (NeuAc-Gal-ceramide) by sugar composition and FAB mass analyses. The ceramide portion of GM4 was composed of a sphingenine (d18:1) as the major sphingoid base, and 2-hydroxy long chain fatty acids, such as 2-hydroxy C24:1, 2-hydroxy C24:0, and 2-hydroxy C22:0. Interestingly, *V. trachuri* was found to bind GM4 of intestine not only from fish but also from chickens, although the bacterium did not bind to GD1a, GM1a, GM2, GM3, GlcCer, and LacCer.

(283) **The Murine Inhibitory Receptor mSiglec-E is Expressed Broadly on Cells of the Innate Immune System Whereas mSiglec-F is Restricted to Eosinophils**

Jiquan Q. Zhang[1], Bj^rn Biedermann[2], Lars Nitschke[2] and Paul R. Crocker[1]

[1] Division of Cell Biology and Immunology, The Wellcome Trust Biocentre at Dundee, University of Dundee, Dundee, GB., [2] Institute of Virology and Immunobiology, University of W¸rzburg, W¸rzburg, Germany.

Murine (m) Siglec-E and mSiglec-F are recently discovered murine sialic acid-binding Ig-like lectins with tyrosine-based inhibitory signaling motifs. They are postulated to be the orthologs of human (h) siglec-7, -8 or -9 and siglec-5, respectively. We report here the first detailed characterization of mSiglec-E, and compare its expression pattern with mSiglec-F. Similar to hSiglec-7, mSiglec-E preferred α 2-8-linked disialic acid over α 2-3- and α 2-6-linked sialic acids. Using a specific Ab, FACS analysis demonstrated that mSiglec-E was expressed mainly on neutrophils in blood and their immature precursors in bone marrow. mSiglec-E was present on peritoneal cavity macrophages and on subsets of mature NK cells and splenic dendritic cells. mSiglec-E was also found on a novel population of peritoneal cavity B-1a-like cells and a subset of splenic B cells enriched in transitional T2 and marginal zone B cells. In striking contrast to mSiglec-E, mSiglec-F was expressed predominantly on eosinophils in blood and their precursors in the bone marrow. The distinct and largely non-overlapping expression profiles of mSiglec-E and mSiglec-F suggest that they play non-redundant roles in the innate immune system. mSiglec-E is likely to modulate the functions of several types of effector cells, whereas mSiglec-F is likely to be more restricted to eosinophil biology.

(284) **Activation-dependent Recruitment of Trimeric G protein Goα to Lipid Rafts in Rat Cerebellar Neurons**

Kohei Yuyama[1,2], Naoko Suzuki[1,2], Yutaka Sanai[1] and Kohji Kasahara[1,2]

[1] Tokyo Metropol. Inst. Med. Sci., [2] PRESTO, JST.

Lipid rafts are subdomains of the plasma membranes that contain high concentrations of glycosphingolipids and cholesterol. The presence within lipid rafts of a variety of proteins, especially those involved in cell signaling, has led to the consensus that these domains play an important role in signal transduction. In the mammalian nervous system, gangliosides, sialic acid-containing glycosphingolipids, are particularly abundant and are involved in the various neuronal activities. However, the molecular mechanism underlying the ganglioside-dependent neural functions remain obscure. To clarify ganglioside and lipid raft-mediated signal transduction, we examined the association of gangliosides with specific signaling proteins in the rat central nervous system. Protein kinase activity was detected in precipitates with monoclonal antibody to ganglioside GD3 from membrane fraction of rat brain. Several phosphorylated proteins of 40, 53, 56, and 80 kDa were isolated. Of these proteins, p53/56 were identified as two isoforms of Src family kinase Lyn and p80 as Csk-binding protein, as we reported previously. In this study, we identified p40 as heterotrimeric G protein α subunit (Goα), based on immunoprecipitation with anti-Goα antibody and pertussis toxin (PTX)-dependent ADP ribosylation. The identification was conformed using a cDNA expression system in CHO cells. In co-transfection with GD3 synthase and Goα expression plasmids, anti-GD3 antibody co-immunoprecipitated Goα. Next, we examined whether Goα exists in lipid rafts.Å@The traditional method of preparation of detergents-resistant lipid rafts was used. The crude membrane fraction of rat cerebellum was lysed in cold buffer containing 0.5% Triton X-100 and the rafts were isolated by flotation in a 5 to 30 % linear sucrose density gradient where distribute in a top few fractions of the gradient. At a result, interestingly, Goα was detected in lipid raft fractions of only developing (postnatal day 4-7) cerebellum, but not of adult one. Then, we examined the difference of Goα affinity for lipid rafts due to the state of Go activity. Heterotrimeric G proteins, Gαβγ, which are activated by stimuli such as ligand-binded receptors dissociates into the GTP-bound Gα and the partner, Gβγ. In the presence of GDPβS or PTX, Goα was associated with Gβγ subunits, and both α and βγ subunits were observed in non-raft fractions. In contrast, addition of GTPγS induced the disassociation of α and βγ subunits, and only α subunit was recruited into raft fractions. Treatment of mastoparan, an activator of Go/i, and stromal cell-derived factor 1α (SDF-1α), a physiological ligand for G protein-coupled receptor involved in chemotaxis of cerebellar granule neurons, induced the activation of Go and promoted the recruitment of Goα to lipid rafts. Moreover, we have shown that Goα was highly concentrated in growth cones of rat developing cerebellar neurons. Growth cone collapse was observed after stimulation of mastoparan and SDF-1α to cerebellar culture neurons, and pretreatment of PTX prevented the growth cone collapse. These results suggest that lipid rafts might play an important role in signal transduction *via* Go on early stage of cerebellar development.

(285) **The Mouse Na^+/K^+-ATPase β1-Subunit Contains a K^+-dependent Cell Adhesion Activity to β-GlcNAc-terminating Glycans**

Kiyoshi Furukawa[1], Masahiko Ikekita[2], Takeshi Sato[1], Yoshihiro Akimoto[3], Hayato Kawakami[3] And Noriaki Kitamura[1,2]

[1] Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, [2] Faculty of Science and Technology, Tokyo University of Science, [3] Department of Anatomy, Kyorin University School of Medicine.

Glycoproteins from mammalian brains contain N-linked oligosaccharides terminating with β-N-acetylglucosamine (GlcNAc) residues in relatively higher proportions in contrast to those from other tissues. The presence of GlcNAc residues at the non-reducing termini of N-linked oligosaccharides may have some biological functions in the brain. In an effort to elucidate the biological functions of such unique oligosaccharides, we have demonstrated that the growth and neurite extension of mouse primary cultured neural cells and mouse and human neuroblastoma cells are regulated through their GlcNAc-terminating oligosaccharides expressed at the cell surface by culturing them in dishes coated with *Psathyrella velutina* lectin (PVL), a β-GlcNAc-binding lectin, [Kitamura et al. (2004) J. Neurosci. Res. 75, 384-390]. These results suggest that GlcNAc-binding

proteins are present at the cell surface and/or extracellular matrices and are involved in cellular interactions. In order to examine this possibility, we isolated a β-N-acetylglucosamine (GlcNAc)-binding protein from adult mouse brain. The solubilized plasma membrane proteins were applied to a GlcNAc-Agarose column, and the bound proteins were eluted with 20 mM chitobiose. A major 48 K protein in the bound fraction visualized after Western blot analysis was subjected to protein sequence analysis. The N-terminal ten amino acid residues showed that the protein to be a mouse Na^+/K^+-ATPase β1-subunit. The fused FLAG-β1-subunit was expressed in Sf-9 cells by gene transfection, and unmodified 30 K and glycosylated 38 K and 40 K proteins were obtained by anti-FLAG antibody column chromatography. When applied to a GlcNAc-Agarose column, only the 38 K and 40 K proteins bound to the column. In the absence of KCl, little of the recombinant proteins bound to a GlcNAc-Agarose column but the 38 K and 40 K proteins bound in the presence of KCl at the concentrations above 1 mM. Inclusion of anti-β1-subunit antibody or 10 mM chitobiose in cell aggregation assays using mouse neural cells and neuro-2a cells resulted in inhibition of cell aggregation. These results indicate that the Na^+/K^+-ATPase β1-subunit is a novel potassium-dependent lectin that binds to GlcNAc-terminating oligosaccharides: it may be involved in neural cell interactions. The importance of GlcNAc-terminating N-linked oligosaccharides has been realized through the defects in organ formation, especially neural tube formation and vascularization and left-right asymmetoric body planning, and resultant embryonic death in the GlcNAcT I-kockout mice, in which most oligosaccharides are of the oligomannose-type [Ioffe and Stanley (1994) Proc. Natl. Acad. Sci. USA 91, 728-732; Metzler et al. (1994) EMBO J. 13, 2056-2065]. Although the biochemical reason that GlcNAc residues in the outer chain moieties of N-linked oligosaccharides are essential for mouse embryogenesis is still obscure, the present study suggests that the binding of GlcNAc-terminating oligosaccharides to the β1-subunit in embryos is critical for brain development.

(286) **Developmental Regulation of Lipid Raft Signaling in the Rat Cerebellum**

Naoko Suzuki[1,2], Kohei Yuyama[1,2], Yutaka Sanai[1] and Kohji Kasahara[1,2]
[1] Tokyo Metropol. Inst. Med. Sci., [2] PRESTO, JST.

Recent studies have indicated that the plasma membrane contains dynamic microdomains that are enriched with cholesterol and glycosphingolipids, resistant to detergent extraction, and of lower density than bulk plasma membrane. These dynamic microdomains are known as lipid rafts and enrich with signaling molecules including src-family kinases and glycosylphosphatidylinositol (GPI)-anchored proteins. Therefore lipid rafts are believed to provide suitable microenvironment to enable selective protein-to-protein interactions as well as local initiation of signal transduction.

Brains and nervous systems are known to be relatively rich in ganglioside, the glycosphingolipid that contains sialic acid in its molecule. As its composition changes dynamically during the brain development, it has been suggested that ganglioside plays crucial roles in synaptogenesis and process elongation. In order to study the function of gangliosides in the central nervous system, we have identified proteins that interact with a ganglioside by using immunoprecipitation with anti-ganglioside antibody.

We have shown that anti-ganglioside GD3 antibody coimmunoprecipitated GPI-anchored neuronal cell adhesion molecule TAG-1, src-family kinase Lyn, Csk (C-terminal src kinase) binding protein Cbp, and trimeric G protein Goα. Furthermore, we have demonstrated that anti-GD3 antibody treatment resulted in activation of Lyn that was present in the lipid raft fraction of cultured rat cerebellar granule neurons. Activation of Lyn has been also observed when TAG-1 on the granule cells were cross-linked with anti-TAG-1 antibody. Since GD3 is already present in early stages of brain development, we hypothesized that signaling through these interacting molecules were subject to developmental regulation. Western blotting analysis showed that total tyrosine (Tyr) phosphorylation level was higher in the developing cerebellum (postnatal day 4) than that in the adult one, and the phosphorylated proteins were highly accumulated in the lipid raft fraction of the developing cerebellum. Therefore, we compared phosphorylation level of Lyn and Cbp in the developing cerebellum (postnatal day 4-7) and that in the adult cerebellum. Active form of Lyn and the Tyr-phosphorylated form of Cbp, that is capable of binding to Csk, were highly accumulated in the lipid raft fraction prepared from the developing cerebellum compared to the lipid raft fraction of the adult one. Overexpression of Lyn and Cbp in CHO cells resulted in phosphorylation of the Cbp, which indicates that Cbp is a substrate of Lyn. In addition, TAG-1, Lyn, Cbp and Goα were highly concentrated in the growth cone fraction prepared from the developing cerebellum. These results suggest that these signaling molecules functionally associate with the lipid rafts on growth cones in developing cerebella.

(287) **The Roles of Hepatitis C (HCV) E2 Glycoprotein Glycosylation in Immune Responses**

Xiao-Lian Zhang[1,2], Qi Wan[1], Yong Feng[1,2], Wei Ju[1], Xinwen Chen[3], Jianguo Wu[2] and Zhongtian Qi[4]
[1] Department of Immunology, College of Medicine, Wuhan University, Wuhan 430071, P.R. China, [2] Ministry of Education Key Laboratory of Virology, College of Life Sciences, , [3] Key Laboratory of Molecular Virology, Wuhan Institute of Virology, , [4] Department of Microbiology, Second Military Medical University, Shanghai 200433, P.R. China.

Glycosylation of viral proteins have been shown to influence antigenic epitopes and modulate immune response. We investigated the role played by N-glycans in the immunogenicity of hepatitis C virus (HCV) E2 envelope glycoprotein, which is heavily modified by N-linked glycosylation. Previous studies showed that the epitopes aa481-500 and aa551-570 of E2 might be important for immunoreactive B-cell domains and that the binding site of E2 for hCD81 is located at aa480-493 and aa544-551 within E2 protein. Here, we investigated the effects on immunological responses by two glycosylation sites (N560YT and N576ST) of E2, which were close to these important regions. Three plasmids encoding E2 mutants were constructed, in which the two N-glycosylation sites (N560YT and N576ST) were mutated separately or in combination. Immunogenicity of these glycosylation mutants of E2 were analyzed in BALB/c mice and compared with that of the wild-type E2 protein using a DNA-based vaccination approach. All mutants decreased both humoral and cellular immune responses induced by the HCV E2 protein compared to that of wild type E2. Among them, the M2 mutant with mutation in the N-linked glycosylation site (N576ST) of E2 induced lowest level of cellular and humoral response in mice in terms of seroconversion rates and antibody productions. These results showed that N-linked glycans of E2 were required for the enhancement of immune response to the HCV E2 protein, especially for antibody response. Our works also provided insights into the development of glycosylated peptides or epitopes as potential vaccines against HCV infection.

(288) **Heparan Sulfate Proteoglycans are Present in Detergent-resistant Membranes Isolated from a Rat Parathyroid Cell Line**

Katarzyna A. Podyma-Inoue[1], Miki Yokoyama[1], Tomoko Kimura[1], Osamu Katsumata[2] and Masaki Yanagishita[1]
[1] Dept. of Hard Tissue Engineering, Biochemistry, Tokyo Medical and Dental University, Tokyo, Japan, [2] Dept. of Physiology, Nihon University School of Dentistry Matsudo, Chiba, Japan.

Heparan sulfate proteoglycans are present on the cell surface and regulate cell functions. They interact with a number of extracellular components and influence, thereby, cell anchorage, shape and growth. Recently, localization of some HSPGs to the lipid rafts, specialized cholesterol- and glycosphingolipid-rich domains has been suggested. The targeting of HSPG to these domains has been postulate to depend on core protein or heparan sulfate (HS) moiety. Using metabolic radiolabeling experiments and sucrose-density gradient ultracentrifugation, we have identified ^{35}S-labeled macromolecule in low density fractions suggesting presence of proteoglycan(s) in lipid rafts isolated from a rat parathyroid (PTr) cell line. Western blot analysis using antibodies recognizing HS-derived epitope, detected the bands of 33 kDa and 150 kDa, suggesting the presence of transmembrane-type proteoglycan, syndecan-1 and other unknown HS-conjugated molecule. Moreover, the content of intact detergent-resistant membranes (DRMs) isolated from cells treated with heparitinase was altered; implying that targeting of other proteins to lipid rafts may be dependent on the presence of HS. Analysis of DRMs, isolated from heparitinase-treated cells, using free-flow electrophoresis, showed cathodal shift when compared to the untreated cells. Also the distribution of DRM markers, i.e., $G_i\alpha$ and CD38, was altered, implying that intact HS may control localization of the proteins within lipid rafts. In this study we demonstrated the presence of HSPGs in lipid rafts in PTr cells. Our data also suggested that HSPG is likely to be important for the organization of DRMs.

(289) **Regulation of Ceramide Kinase by Specific Phosphatidylinositol Phosphates**

Tack-Joong Kim[1], Susumu Mitsutake[1], Tadaomi Takenawa[2] and Yasuyuki

Igarashi[1]
[1] Department of Biomembrane and Biofunctional Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12-jo, Nishi 6-chome, Kita-ku, Sapporo 060-0812, Japan, [2] Department of Biochemistry, Institute of Medical Science, University of Tokyo and Japan Science and Technology Corporation, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan.

Recently, we demonstrated that ceramide kinase (CERK), which catalyzed the formation of ceramide 1-phosphate (C1P), was a mediator of calcium-dependent degranulation in mast cells (Mitsutake *et al., J. Biol. Chem.*, 279, 17570-7). N-terminus of this enzyme contains a pleckstrin homology (PH) domain, a motif for membrane targeting. Up to now, the biochemical function of this PH domain of CERK has not been characterized. In this study, we investigated the function of this PH domain in CERK in relation to phosphatidylinositol phosphates (PtdIns) as known PH domain ligand. In vitro lipids binding assays demonstrated that CERK showed high affinity to PtdIns, and the kinase activity was specifically enhanced by PtdIns-(3,5)-P_2, a PtdIns binds to CERK, in a dose dependent manner. Furthermore, in fractionation experiments using wild type or PH domain of CERK-overexpressing COS7 cells, we showed that both wild type and PH domain were strongly associated with membrane, and the N-terminal PH domain of CERK was needed to exert its kinase activity. These results indicated that CERK plays a crucial role in association of membrane and regulation of activity through binding with phosphatidylinositol phosphates.

(290) **Generation of Specific Monoclonal Antibodies to Murine CD33-related Siglecs Using Phage Display Technology**

Claire Jones[1], Jiquan Zhang[1], Tyrone Bowes[1], Karen Cromie[2], Alistair Henry[2] and Paul Crocker[1]
[1] Division of Cell Biology and Immunology, The Wellcome Trust Biocentre, University of Dundee, Dundee, United Kingdom DD1 5EH, [2] Celltech R&D Ltd, 216 Bath Road, Slough, Berkshire, United Kingdom SL1 4EN.

Siglecs (11 in humans, 8 in mice) are transmembrane sialic acid binding immunoglobulin-like lectins involved in adhesive and signalling functions within the haemopoietic, immune, nervous and reproductive systems. Based on sequence similarity in the extracellular region, siglecs can be divided into two subgroups: a non-CD33-related subgroup and the recently-discovered CD33-related subgroup. There are 8 CD33-related siglecs in humans and 5 in mice. In humans, expression of CD33-related siglecs is highly specific to distinct subsets of haemopoietic cells but less is known about the mouse proteins due to lack of specific mAbs. Here we describe the use of phage display technology to generate specific mAbs against murine CD33-related siglecs. A primary phage library with a complexity of $\sim\sim10^{11}$ inserts was used to select for antigen-specific single chain (scFv) antibody fragments by panning on CD33-related siglec-Fc proteins coated on plates. Through multiple rounds of screening, a panel of scFv clones against each mSiglec-Fc was identified and shown to be specific by ELISA. Biacore was used to measure the affinities of selected scFv fragments which were found to be in the pM to nM range. The VH and VL regions of identified selected scFvs were re-derived into a whole antibody framework (isotype IgG1k) and the binding specificity for each mAb was confirmed by FACS using stably transfected CHO cell lines and also on primary mouse cells of the bone marrow, spleen, blood and peritoneal cavity. The panel of mAbs will provide useful tools for analysis of the expression and functions of murine CD33-related siglecs.

(291) **Analysis of the Specificity and Sites of Action of Lunatic Fringe on Glycosylation and Signaling of Notch1**

Raajit Rampal, Sameer Khanijo, Annie Li and Robert S. Haltiwanger
Department of Biochemistry and Cell Biology, Institute for Cell and Developmental Biology Stony Brook University, Stony Brook, NY 11794-5215..

The Notch protein is a transmembrane receptor involved in a wide variety of cell fate decisions in metazoans. Importantly, mutations of the Notch protein and components of its signaling pathway have been implicated in an array of human diseases (CADASIL, T-cell leukemia, multiple sclerosis). In mammals, Notch becomes activated upon binding of its extracellular domain to its ligands Delta and Serrate, which are present on the surface of apposed cells. The extracellular domain of Notch contains 36 tandem Epidermal Growth Factor-like (EGF) repeats. Many of these EGF repeats contain consensus sequences for modification by *O*-fucose. Additionally, some of the *O*-fucose moieties on EGF repeats of Notch can be further elongated by the action of Fringe, a β 1,3-N-acetylglucosaminyltransferase. Three mammalian Fringes have been identified; Manic, Lunatic and Radical Fringe. Modification of Notch by Fringe modifies the response of Notch to its ligands. Not all EGF repeats modified by *O*-fucose are further modified by Fringe. As an example of this, the first EGF repeat of both human clotting factors VII and IX are known to be modified by *O*-fucose. However, *in vivo*, only the *O*-fucose on factor IX is modified by Fringe, even thought both proteins are produced in the same cell. Thus, Fringe selectively modifies *O*-fucose in the context of some EGF repeats, but not others. This specificity appears to be encoded in the amino acid sequence of individual EGF repeats, as the first EGF repeat of factors VII and IX differ by only 12 of 24 amino acids. Preliminary data using factor VII and factor IX expressed in CHO cells suggests that several of these amino acids are important for recognition by Fringe. We therefore seek to ascertain what determines the specificity of Fringe using recombinant human clotting factor VII and factor IX as a model substrate system. We are performing *in vitro* enzyme assays using purified Lunatic Fringe (produced in mammalian cells) with recombinant EGF1 of factor VII and IX, modified with *O*-fucose, as acceptor substrates. Amino acids believed to play a role in recognition by Lunatic Fringe are being mutated, and the altered EGF repeats will be evaluated kinetically as substrates *in vitro*. We also seek to determine which of the glycosylated EGF repeats are most important for Notch signaling. Towards this aim, we are adapting a cell-signaling assay that allows us to test the importance of sites of glycosylation of Notch on signaling function. Using this assay, we can transiently transfect wildtype Notch or Notch containing mutations in known sites of glycosylation, or on amino acids necessary for elongation of *O*-fucose by Fringe, along with a luciferase reporter into cells. These cells are then co-cultured with cells either expressing no ligand, or expressing a Notch-ligand. The relative-fold activation of Notch can then be determined via a luciferase assay.This work was supported by NIH grant GM61126.

(292) **Oligochitin Elicitor Receptor in Plasma Membrane of Suspension-cultured Rice Cells – Solubilization, Purification and Molecular Cloning**

Hanae Kaku[1], Yuki Ito[1], Yoko Nishizawa[1], Naoshi Dohmae[2], Koji Takio[3], Naoko Ishii-Minami[1], Ayako Katou[1], Eiichi Minami[1] and Naoto Shibuya[1,4]
[1] NIAS, Tsukuba, 305-8602, Japan, [2] RIKEN, Wako, 351-0198, Japan, [3] RIKEN, Mikatsuki-town, 679-5148, Japan, [4] Meiji University, Kawasaki, 214-8571, Japan.

Plants evoke a set of defense responses upon infection with pathogens. It is generally considered that there is an exchange of signals between plants and pathogens that triggers a set of defense responses in plants. Similar responses are observed when plants are treated with elicitors derived from cellular components of the host or pathogens One such elicitor, N-Acetylchitooligosaccharide, is highly potent at subnanomolar level in suspension-cultured rice cells, inducing a set of defense-related responses such as membrane depolarization, change of ion fluxes, production of reactive oxygens, expression of defense-related genes and production of phytoalexins (1). The elicitor activity is strictly dependent on the structure of N-acetylchitooligosaccharides, that is, N-acetylchitoheptaose and octaose show the highest activity whereas hexamer or shorter ones and their deacetylated form, chitooligosaccharides, show much lower activity. These results strongly indicated that the elicitor signal is mediated by the specific receptor that is likely to be localized in the plasma membrane (PM) of rice. Using chemically modified N-acetylchitooligosaccharide as a probe, we have identified a high affinity binding protein (CEBiP; Oligochitin Elicitor Binding Protein) in the PM of rice cells (2). The dependency of the binding affinity of the protein on the structure of the elicitor was very similar to that of the elicitor activity, indicating that the protein be a receptor of N-acetylchitooligosaccharide elicitor. We have found similar proteins in the PM fractions of other monocot and dicot plants, in good correlation with the elicitor activity of N-acetylchitooligosaccharides (3). It is, therefore, likely that the recognition system of N-acetylchitooligosaccharide is common to higher plants. We are going to show the recent results on the purification of CEBiP and its role in the elicitor signaling in rice. 1) Shibuya, N. and Minami, E. Physiol. Mol. Plant Pathol., 59, 223-233 (2001) 2) Ito, Y. et al., Plant J., 12, 347-356 (1997) 3) Okada, M. et al., Plant Cell Physiol., 43, 505-512 (2002)

(293) **Functional Implication of Intracellular Mannan-binding Protein**

Motohiro Nonaka[1], Bruce Y. Ma[1], Misato Ohtani[1], Keiko Miwa[1], Tomoaki Nakagawa[1], Shogo Oka[1], Nobuko Kawasaki[2], Akitsugu Yamamoto[3] and Toshisuke Kawasaki[1]

[1] Department of Biological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Japan, [2] School of Health Sciences, Faculty of Medicine, Kyoto University, Japan, [3] Nagahama Institute of Bio-Science and Technology, Japan.

Mannan-binding proteins (MBPs) are C-type mammalian lectins naturally occured in two forms, serum MBP (S-MBP) and liver MBP (L-MBP). Human S-MBP and L-MBP are translated from a single mRNA. S-MBP activates complement in association with MBP-associated serine proteases (MASPs) via the lectin pathway. The functions of L-MBP are poorly known. To investigate subcellular L-MBP functions, we expressed human MBP cDNA in human hepatoma and small intestine cells. The expression of MBP reproduced the native MBP differentiation of S-MBP and L-MBP in these two different cell lines. The subcellular localization of L-MBP was studied using green fluorescent protein (GFP) fusions and confocal microscopy. L-MBP showed distinct accumulations in cytoplasmic granules, and partially localized in ER and predominantly in cis/trans Golgi. L-MBP was also found to predominantly localize in coatomer protein I (COP I) vesicles, which serve as retrograde Golgi-to-ER transport for the continuous recycling of escaped and misfolded ER resident proteins. The unique intracellular distribution of L-MBP would disappear and display equally a diffuse cytoplasmic localization after tunicamycin treatment. Cycling of L-MBP is suggested by colocalization with retrograde cargo trapped in Golgi apparatus and modification of the N-linked carbohydrate by glycosylation enzymes of medial Golgi cisternae. Using coimmunoprecipitation of MBP and a cargo glycoprotein from transfected cells, we demonstrate an interaction between MBP and the cargo in vivo. The interaction was mediated via high mannose-containing asparagines-linked oligosaccharides. Our findings suggest that the intracellular L-MBP may play an important role in glycoprotein quality control as a lectin chaperone.

(294) Characterization of Inhibitory Signaling Motifs of Natural Killer Cell Receptor, Siglec-7: Attenuated Recruitment of Phosphatases by the Receptor is Attributed to Two Amino Acids in the Motifs

Toshiyuki Yamaji, Takane Teranishi and Yasuhiro Hashimoto

Glyco-chain Functions Laboratory, RIKEN Frontier Research System, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, JAPAN.

Leukocyte activation is controlled by the balance of two groups of receptors that are functionally opposite; i.e., activating and inhibitory receptors. The engagement of activating receptor induces rapid tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail to transduce activating signals. When an inhibitory receptor co-engages with an activating receptor, tyrosine residue in the immunoreceptor tyrosine-based inhibitory motif (ITIM) of the inhibitory receptor is phosphorylated. The resulting phosphotyrosine recruits SH2-containing phosphatases such as SHP-1 and SHP-2, which dephosphorylate several signaling molecules, resulting in inhibition of the activating signals induced by ITAM-bearing receptors. Siglec-7 is a sialic acid-binding lectin recently identified as an inhibitory receptor on natural killer cells and monocytes. We previously characterized sugar-binding specificity of Siglec-7, and found that the lectin preferably binds a unique oligosaccharide structures such as sialyl-alpha2,8-sialyl and galactosyl-beta1,3-[sialyl alpha2,6]-hexosaminyl residues (1, 2). The cytoplasmic domain of Siglec-7 contains two signaling motifs: a membrane-proximal ITIM (Ile435-Gln-Tyr-Ala-Pro-Leu440) and a membrane-distal one (Asn458-Glu-Tyr-Ser-Glu-Ile463). To investigate whether the two motifs contribute to the inhibitory signaling of Siglec-7, we replaced Tyr437 and/or Tyr460 with phenylalanine. The tyrosine mutants or wild-type Siglec-7 were transfected to human monocytic U937 cells, which endogenously expressed an activating receptor (Fc-gamma receptor I) for calcium mobilization. Co-ligation of wild-type Siglec-7 with the Fc receptor markedly reduced calcium mobilization, indicating the inhibitory effect of Siglec-7. Simultaneous substitution of both tyrosines almost abolished this effect. Replacement of Tyr460 partially reduced the inhibitory activity, whereas replacement of Tyr437 did not reduce inhibitory activity, suggesting strong dependency of Siglec-7 signaling on the distal tyrosine. We also demonstrated that, upon pervanadate treatment, Siglec-7 recruited the protein tyrosine phosphatases SHP-1 and SHP-2 less efficiently than did other inhibitory receptors such as Siglec-9 and leukocyte-associated Ig-like receptor. Mutational analysis revealed that the inefficient recruitment of the phosphatases by Siglec-7 was mainly due to Pro439 in the Y+2 position of the proximal motif and Asn458 in the Y–2 position of the distal motif. These amino acids appeared to affect not only phosphatase recruitment but also the subsequent attenuation of Syk phosphorylation. MHC-recognizing inhibitory receptors recruit the phosphatases much more efficiently than does Siglec-7 and play a key role in the discrimination between self and non-self; i.e., aberrant cells that fail to express MHC molecules are susceptible to NK cell-dependent killing (missing-self recognition). Nevertheless, some tumor cells such as melanoma cells that occasionally express low levels of MHC molecules still survive and form clinically apparent tumors, suggesting that these tumor cells have some mechanism for escaping NK cell-dependent killing. It is tempting to speculate that Siglec-7-dependent inhibition is involved in the MHC-independent mechanism. Indeed, melanoma cells often express GD3 ganglioside, a potent ligand for Siglec-7. The ligand may transduce a Siglec-7-dependent inhibitory signal to NK cells for tumor survival in vivo. Modulation of Siglec-7 signaling may lead to a new therapeutic means against tumor cells in the future. (1) T. Yamaji et al. J Biol Chem, 277, 6324, 2002 (2) T. Yamaji et al. Methods in Enzymol, 363, 104, 2003.

(295) Mouse T cell CD166 is a Disialic Acid-containing Glycoprotein and Involved in T Cell Activation

Chihiro Sato[1,2,3], Keiko Nohara[4], Tsukasa Matsuda[1] and Ken Kitajima[1,2,3]

[1] Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, [2] Laboratory of Animal Cell Function, Nagoya University Bioscience and Biotechnology Center, [3] Institute for Advanced Research, Nagoya University, Nagoya 464-8601, Japan , [4] Environmental Health Sciences Division, National Institute for Environmental Studies, Tsukuba 305-8506, Japan .

The disialic acid (diSia)-containing glycolipids are known to be involved in various biological processes. In contrast to the glycolipids, little attention has been paid to the occurrence and functions of the diSia in glycoproteins. Recently, highly sensitive chemical and immunochemical methods to detect di-, oligo-, and polySia residues in glycoproteins have been developed and the frequent occurrence of the di/oligo/polySia-containing glycoproteins is demonstrated using these methods (1). We also identified several glycoproteins containing the di/oligo/polySia (2, 3). To gain an insight into the biological function of diSia residues in glycoprotein, we focused on the diSia epitope on mouse T cells. It has been demonstrated that a ganglioside species containing tandem sialic acid dimer is predominant in rat T cells. A monoclonal antibody, which specifically recognizes the diSia epitope, enhances CD3-ligated and CD4-ligated T cell activation through the binding to the diSia epitope not only on gangliosides but also on the 100 kDa-glycoprotein in rat.

In this study, we demonstrated that the diSia-containing 100 kDa-glycoprotein also occurred in mouse T cells and first identified the 100 kDa-glycoprotein as CD166 (ALCAM, activated leukocyte cell adhesion molecule). We also showed that T cell CD166 contained the diSia epitope on O-linked glycans, and that it was colocalized with the diSia-containing gangliosides in rafts, membrane microdomains on the cell surface. The expression of diSia on CD166 was up-regulated during CD3-ligated and CD3-/CD4-ligated T cell activations. Furthermore, we demonstrated the enhancement of the CD3-ligated or CD3-/CD4-ligated T cell activation by the anti-diSia antibody in mouse T cell. We also demonstrated that the expression of CD166 and ST8Sia mRNAs were up-regulated during CD3-ligated and CD3-/CD4-ligated T cell activation in the presence of the anti-diSia antibody. Taken together, it is indicated that CD166 is a diSia-containing glycoprotein not only in mouse brain (3) but also in mouse T cells and that the diSia epitope on CD166 may be involved in T cell activation in cooperation with that of gangliosides.

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(296) Glyco-receptor for Gangliosides and GalNAc-terminated Oligosaccharides to Induce cdc42-mediated Neuronal Development

Hideyoshi Higashi[1,2] and Yoshio Hirabayashi[1]

[1] Neuronal Circuit Mechanisms Research Gr., Brain Science Institute, RIKEN, Wako, Saitama, Japan, [2] CREST, JST, Kawaguchi, Saitama, Japan.

Extracellular glyco-chains are recognized by receptor molecules to induce intracellular signaling. We recently demonstrated two different glyco-signals that lead cytoskeletal actin reorganization to form filopodia and neuronal dendrite generation. The one is GT1b/GD1b-CaMKII signal consisting of Ca^{2+} release from intracellular store, CaMKII activation and cdc42 activation. The other is GM2/GalNAc-PKA signal consisting of

cAMP production by adenylate cyclase, PKA activation and cdc42 activation. Exposure of neuronal cells to nanomolar concentrations of these gangliosides or oligosaccharides trigger either of the events suggesting contribution of a cell surface glyco-receptor. The common critical molecule, cdc42 is a Rho family GTPase that facilitates cytoskeletal actin reorganization to form filopodia and mediates neuronal dendritogenesis. The upstream signaling cascade of cdc42 is not clearly characterized except in Swiss 3T3 fibroblast where bradykinin (BK) is the inducer of the filopodia.

To test whether BK receptor, B2 is the glyco-receptor, we have examined the effects of B2 antagonist on the signals and the glyco-receptor activity of B2 expressing yeast. BK is 9-amino acid-peptidic hormone derived from a plasma protein, kininogen. A potent peptidic B2 antagonist, Hoe140 completely inhibited both glyco-signals, CaMKII or PKA activation, and filopodia formation. In case of GT1b/GD1b-CaMKII signal, we successfully constructed the glyco-signaling cascade in a non-neuronal cell by overexpression of B2 in NIH3T3 fibroblast. B2 is a seven-transmembrane G protein-coupled receptor (GPCR). In vertebrates, we have 200-300 GPCRs including uncharacterized "orphan" receptors. Some GPCRs form homo- or hetero-dimers to regulate the receptor signals. To further confirm that B2 receptor is the glyco-receptor, we have used a much more simple cell system, yeast, *Saccharomyces cerevisiae.* Yeast has only two GPCRs and both of them are characterized. One of the GPCR, ⊃)-factor receptor transduces the pheromone signal to yeast G-protein, GPA and this activates several protein kinases and a pheromone-responsive transcription factor leading conjugation. Although GPA itself cannot react with mammalian GPCR, GPA containing 5 C-terminal amino acid residues of mammalian GPCR can react with mammalian GPCR. Thus, the yeast expressing the chimera G-protein and mammalian B2 receptor can transduces BK signal to the pheromone-responsive transcription factor. We used the yeast system with ⊃↵-galactosidase as the reporter. The yeast expressing B2 responded to GT1b, GM2, their oligosaccharides and GalNAc(⊃)1-4)decamer. The results indicate that B2 receptor is the glyco-receptor for GT1b and GD1b, as well as GM2 and GalNAc-terminated oligosaccharides. In brain, B2 receptor is distributed ubiquitously and selectively in neurons. The glyco-signal possibly plays a role in dendrite development, maturation and synapse formation.

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(297) Membrane Redistribution of Gangliosides and GPI-anchored Proteins Under Conditions of Lipid Raft Isolation

Marija Heffer-Lauc[1], Gordan Lauc[2], Leonardo Nimrichter[3], Susan E. Fromholt[3] and Ronald L. Schnaar[3]

[1] University of Osijek School of Medicine, Department of Biology, J. Huttlera 4, 31000 Osijek, Croatia, [2] University of Osijek School of Medicine, Department of Chemistry and Biochemistry, J. Huttlera 4, 31000 Osijek, Croatia, [3] Departments of Pharmacology and Neurosciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

Gangliosides, GPI-anchored proteins, and certain signaling molecules segregate from bulk membrane lipids into lateral domains termed lipid rafts, which are often isolated based on their insolubility in cold non-ionic detergents. During immunohistological studies of gangliosides we found that cold Triton X-100 solubility is bidirectional, leading to histological redistribution from brain gray to white matter. When brain sections were treated with ≥0.25% Triton X-100 at 4∞C, ganglioside GD1a, which is normally enriched in gray matter and depleted in white matter, redistributed into white matter tracts. Incubation of brain sections from knockout mice lacking GD1a with wild type sections in the presence of cold Triton X 100 resulted in GD1a redistribution from wild type gray matter to knockout white matter. GM1, which is normally enriched in white matter, remained in white-matter after cold detergent treatment and did not migrate to knockout mouse brain sections. However, when gray matter gangliosides were enzymatically converted into GM1 *in situ*, the newly formed GM1 transmigrated to knockout mouse brain sections in the presence of cold detergent. When purified GD1a was added to knockout mouse brain sections in the presence of cold Triton X-100 it preferentially incorporated into white matter tracts. These data demonstrate that brain white matter is a sink for gangliosides, which redistribute from gray matter in the presence of low concentrations of cold Triton X-100. A glycosylphosphatidylinositol (GPI) anchored protein, Thy-1, also transmigrated from wild type to Thy-1 knockout mouse brain sections in the presence of detergent at 4∞C, although less efficiently than did gangliosides. These data raise technical challenges for using non-ionic detergents in certain histological protocols and for isolation of lipid rafts to study gangliosides and GPI-anchored proteins in the brain.

(298) EGF-induced Mobilization of Neu3 Sialidase to Membrane Ruffles in A431 Cells

Kazunori Yamaguchi, Keiko Hata, Tadashi Wada, Setsuko Moriya and Taeko Miyagi

Division of Biochemistry, Miyagi Cancer Center Research Institute and CREST, JST, Japan.

Sialidase reaction is an initial step for degradation of the sialo-glycoconjugates and thought to be important to many biological phenomena. Recent studies suggest that mammalian sialidases are important not only in lysosomal degradation process but also in many cellular processes. Our previous biochemical characterization and recent progress in molecular cloning of mammalian sialidases have revealed the existence of at least four forms with different substrate preferences and subcellular localization in the cells. Of these sialidases, a ganglioside-specific sialidase, Neu3, is intriguing because of its strict ganglioside preference and involvement in various biological phenomena including neuronal differentiation (1), insulin signaling (2), and tumorigenesis (3). Although Neu3 is thought to be associated with plasma membrane, the functional cellular localization is not fully determined.

To obtain further insights into understanding of its function, we examined the subcellular localization of the human ortholog (NEU3) by indirect immunofluorescence microscopy. The HA-tagged human NEU3 was exogenously expressed in HeLa and A431 cells by using adenovirus-mediated transduction and probed by anti HA antibody or anti human NEU3 monoclonal antibody. Under a normal growth condition, a fraction of expressed NEU3 was localized at leading edge of plasma membrane. The cell-peripheral distribution was diminished when the cells were starved by serum deprivation. EGF-treatment of starved A431 cells induced the recruitment of NEU3 at ruffling cell periphery within 5 min, co-localized with marked phalloidin staining. A part of recruited NEU3 was colocalized with Rac and GM1 staining. Methyl-β-cyclodextrin treatment inhibited EGF-induced cell ruffling and also peripheral distribution of NEU3. After 15 min of the treatment cells showed decreased ruffling activity accompanied by decreased peripheral distribution of NEU3. Cells overexpressing NEU3 showed enhanced motility in the assay employing micropore membrane filter. These results suggest that NEU3 may alter its functional location in the plasma membranes in response to cellular signaling, leading to functional involvement in various cellular events including cell motility.

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(299) Lectin Mapping of Adult Rat Hippocampus Shows No Difference After an Avoidance Learning Task.

Alejandra Hidalgo[1], Valeria Burgos[1], HaydeÈ Viola[2], Jorge Medina[2] and Pablo Argibay[1]

[1] Instituto de Ciencias B·sicas y Medicina Experimental del Hospital Italiano de Buenos Aires - Argentina, [2] Instituto de Biolog'a y Neurociencias Profesor Dr Eduardo de Robertis ,Facultad de Medicina UBA - Argentina.

The carbohydrate chains of glycoproteins, glycolipids and proteoglycans play an important role in numerous cell processes such as cell-to-cell and cell-to-matrix interactions. Changes in cell surface carbohydrate structures have been observed during development, and under normal and pathological conditions. A number of studies indicate that glycoconjugates are involved in the modulation of the synaptic connections of the hippocampus in processes such as learning and memory. However, no lectin-cytochemistry was performed in these studies. Objective: To study

the hippocampal distribution of glycoconjugates of adult normal rats subject to an inhibitory avoidance task. Methods: On the training session, male Wistar rats were placed on a platform facing a grid where they received a scrambled foot shock immediately upon stepping down. This inhibitory task, when increased in latency, is known to involve the specific repression of a natural exploratory tendency of the rat to go beyond the platform, being this behaviour mainly hippocampal-dependent. Animals were divided in three experimental groups: those trained in the avoidance task (Trained), those placed directly onto the grid where they immediately received the foot shock (Shocked), and finally those never exposed to the apparatus (Naive). Animals were sacrificed 6 h post training; brains were fixed in Bouin and processed for paraffin embedding. The expression of glycans in the hippocampus were examined by lectin-cytochemistry using the following sugar specific lectins: VVL (terminal alpha/beta N-Acetylgalactosamine), GNL (terminal Mannose alpha-1,3), PNA (Galactose beta-1,3 N-Acetylgalactose), ECA (Galactose beta-1,4 N-Acetylglucosamine), SNA (Sialic Acid alpha-2,6 Galactosamine), MAL II (Sialic Acid alpha-2,3), WGA (terminal N-Acetylglucosamine with/without Sialic Acid), sWGA (terminal N-Acetylglucosamine without Sialic Acid), GSL II (terminal alpha/beta-N-Acetylglucosamine). Results: Galactose beta-1,4 N-Acetylglucosamine (ECA lectin), terminal N-Acetylglucosamine with/without Sialic Acid (WGA lectin), terminal N-Acetylglucosamine without Sialic Acid (sWGA) and terminal a-b-N-Acetylglucosamine (GSL II) were negative throughout the hippocampus. On the contrary, in the alveus and neuropil of the polymorph layer of the dentate gyrus there was an intense staining of Galactose beta-1,3 N-Acetylgalactose (PNA lectin). Some CA1 pyramidal neurons showed a positive staining for terminal alpha/beta N-Acetylgalactosamine (VVL lectin) around the cell body and the first portion of the axonic cone, as well as a marked expression of this sugar in CA1, CA2 and CA3 alveus. Hippocampal pyramidal cells (cytoplasm and the first portion of apical dendrite) as well as dentate gyrus granular layer were positive for Mannose alpha-1,3 as evidenced by GNL lectin staining. Conclusion: In the present study we found no significant change in the glycosylation pattern of hippocampal glycoproteins as a result of the learning task. We suppose that for glycosylation modifications to occur, a longer time interval is needed after the avoidance training. However, our results show that there is an evident patterned distribution of glycans in the rat brain, as manifested by other studies.

(300) Sialic Acid Binding Specificity of CD33-related Siglecs can be Changed via Partial Gene Conversion by Adjacent Pseudogenes

Toshiyuki Hayakawa[1], Takashi Angata[2], Elliott H. Margulies[3], Tarjei Mikkelsen[4], Eric D. Green[3] and Ajit Varki[1]

[1] Glycobiology Research and Training Center, Departments of Medicine and Cellular and Molecular Medicine, University of California at San Diego, La Jolla, CA. , [2] National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan. , [3] Genome Technology Branch and NIH Intramural Sequencing Center, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD. , [4] The Broad Institute, Cambridge, MA..

Siglecs (sialic acid-binding immunoglobulin superfamily lectins) are a family of cell surface receptors involved in regulating the immune response. The CD33-Related Siglecs (CD33rSiglecs, namely Siglec-3, -5 through -11 and -XII in humans) are a subgroup of these molecules found primarily on cells of the innate immune system. All are type-1 transmembrane proteins with an N-terminal sialic acid-recognizing V-set domain followed by a variable number of C-2 set domains, a transmembrane region and a cytosolic C-terminal domain that includes two tyrosine-based signaling motifs. Available data suggest an inhibitory signaling role in the innate immune response, mediated by recognition of host sialic acids as "self". Nine of the 13 known primate Siglec genes along with 14 Siglec pseudogenes comprise the CD33-related Siglec gene cluster on human chromosome 19. Gene conversion is a mechanism for copying part of a genomic sequence into another, contributing to genetic diversity. Pseudogenes are known to play role in generating functional diversity of related genes by causing dramatic sequence change via gene conversion (e.g., antibody diversity in chickens). We recently analyzed genomic sequences of the CD33-related Siglec gene cluster in three primates (human, chimpanzee and baboon) and found evidence for rapid evolution in this gene family (Angata et al., PNAS, in press). Additional evolutionary studies using distance-based phylogenetic trees show evidence for three partial gene conversions between Siglec genes and adjacent Siglec pseudogenes. All three involve the coding regions for extracellular domains that mediate sialic acid recognition, and two represent cases where a pseudogene converted a known Siglec gene in the human or chimpanzee lineage. The converted Siglec genes did not acquire any nonsense mutation during gene conversion and maintained an intact open reading frame. Functional analyses using recombinant proteins showed marked differences in sialic acid-binding properties between the converted Siglecs and the non-converted orthologs. These findings suggest that gene conversion with pseudogenes has contributed to the rapid functional evolution of the Siglecs in the hominoid lineage, and provides a novel mechanism for changing sialic acid binding specificity. We hypothesize that this is another mechanism that allows for rapid evolutionary adjustments in the recognition of endogenous sialic acids as "self" by CD33rSiglecs, a potential factor in controlling the innate immune response.

(301) Structural Analysis of Cytokine Signaling Modulators

Cristina I. Silvescu[1], Emily A. Partridge[2,3], James W. Dennis[2] and Vernon N. Reinhold[1]

[1] Department of Biochemistry and Molecular Biology, Durham, NH, [2] Mount Sinai Hospital, Toronto, ON, [3] University of Toronto, ON, Canada.

Glucose flux through the hexoseamine pathway to UDP-GlcNAc elevates Mgat 5 modifications, poly Nacetyllactoseamine synthesis and glycan content on cytokine receptors. These promote galectin-cytokine receptor interactions and proliferation signals in tumor cells. To determine the effect of hexoseamine pathway on N-glycans biosynthesis we compared Mgat $5^{-/-}$ cells in normal growth conditions to Mgat $5^{-/-}$ in GlcNAc supplemented medium and to Mgat $5^{+/+}$ cells grown in normal or supplemented medium. The *N*-linked glycan analysis shows an increase of complex glycan concentrations comparative to the high mannose ones in the GlcNAc supplemented sample. At the same time in the unsupplemented cells the high mannose glycans are more abundant. These data strongly suggest a direct dependence between the UDP-GlcNAc levels and the concentrations of GlcNAc transferases productsÖ.the complex glycans. The absence of Mgat 5 in the null background blocks tetra antennary and polylacNAc glycan synthesis and increased GlcNAc levels determine accumulation of low molecular weight glycans and bisected structures. Our results demonstrate that UDP-GlcNAc flux through hexoseamine pathway influences *N*- glycosylation and glycan content on the surface of receptor proteins modulating cytokine signaling. MS profiles and MS(n) disassembly reveals key fragments ions indicative of specific branching patterns defining pathway of synthesis under the varying metabolic conditions.

Experiments were carried out using a MALDI-IT (Kratos-Shamadzu), LCQ, and LTQ (Thermo) mass spectrometers. Supported by BRIN-NCRR(VR) and NIGMS(VR).

(302) E-selectin Ligands on Human Leukocytes

Leonardo Nimrichter[1,2], Monica M. Burdick[3], Wouter Laroy[1], Mark A. Fierro[1], Sherry A. Hudson[4], Christopher E. Von Seggern[1], Robert J. Cotter[1], Bruce S. Bochner[4], Konstantinos Konstantopoulos[3] and Ronald L. Schnaar[1]

[1] Department of Pharmacology, The Johns Hopkins School of Medicine, Baltimore, MD 21205, [2] Instituto de Microbiologia Prof. Paulo de Goes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [3] Department of Chemical & Biomolecular Engineering, The Johns Hopkins University, Baltimore, Maryland 21218, [4] Department of Medicine, Division of Allergy and Clinical Immunology, The Johns Hopkins University School of Medicine, Baltimore, MD 21224.

E-selectin and P-selectin on activated vascular endothelium mediate the initial steps of inflammation by binding to complementary sialic acid- and fucose-containing carbohydrate ligands on the surface of neutrophils. The human neutrophil ligand for P-selectin is the glycoprotein PSGL-1, however ligands for E-selectin on human neutrophils have yet to be established. The present studies, together with prior published work, establish that pentadeca- and hexadecasaccharide glycosphingolipids characterized as monosialogangliosides with 5 poly-N-acetyllactosamine repeats and 2-3 fucose residues fulfill the criteria for designation as the functional endogenous E-selectin ligands on human neutrophils. This claim is based on the following findings: (1) Most E-selectin binding activity on human (as opposed to mouse) neutrophils is resistant to extensive proteolysis (Kobzdej *et al*., 2002); (2) Treatment of human neutrophils with inhibitors of glycosphingolipid biosynthesis inhibits E-selectin but not P-selectin-mediated adhesion; (3) Isolation of sialylated glycosphingolipids from human neutrophils combined with total glycan analysis, fractionation, quantitative selectin-mediated cell tethering/rolling studies, and mass spectrometry revealed that ~60% of the total tethering capacity was represented by structures with masses consistent with

$NeuAc_1Glc_1Gal_6GlcNAc_5Fuc_{(2-3)}Cer$; (4) Terminally sialylated poly-LacNAc structures consistent with this composition were previously characterized from leukocyte-related human HL-60 cells, and termed 'myeloglycans' (Stroud *et al*., 1996); and (5) The expression levels of the potent E-selectin-binding glycolipids on human neutrophils correlate with the densities required to support E-selectin-mediated tethering and rolling. These observations are consistent with designating the named glycolipids as functional E-selectin ligands on human neutrophils.

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(303) **Glycan Array Screening Reveals a Candidate Ligand for Siglec-8**

Bruce S. Bochner[1], Richard A. Alvarez[2], Padmaja Mehta[3], Nicolai V. Bovin[4], Ola Blixt[5], John R. White[6] and Ronald L. Schnaar[7]

[1] Division of Clinical Immunology, Department of Medicine, The Johns Hopkins School of Medicine, Baltimore, MD 21224, [2] Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, [3] Oklahoma Medical Research Foundation, Cardiovascular Biology Research Program, Oklahoma City, OK 73104, [4] Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia, [5] Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037, [6] GlaxoSmithKline, King of Prussia, PA 19406, [7] Department of Pharmacology and Molecular Sciences, The Johns Hopkins School of Medicine, Baltimore, MD 21205.

Sialic acid binding immunoglobulin-like lectin 8 (Siglec-8) is expressed on human eosinophils, basophils, and mast cells, where it may regulate their function and survival. Previously published studies demonstrated sialic acid-dependent binding of Siglec-8, but failed to reveal significant sub-structure specificity or high affinity of that binding. In an effort to test a broader range of potential ligands, a Siglec-8-Fc chimeric protein was tested for binding to ~180 different glycan structures immobilized as biotinylated glycosides on a 384-well streptavidin-coated plate. Of these, ~40 structures were sialylated, including structures with 2-3, 2-6 and 2-8 linked sialic acids in various glycan sequences. Among these, avid binding was detected to a single defined glycan: NeuAcα2-3(6-O-sulfo)Galβ1-4[Fucα1-3]GlcNAc, also referred to in the literature as 6'-sulfo-SLe^x. Notably, neither unsulfated SLe^x (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc) nor an isomer with the sulfate on the 6-position of the GlcNAc residue (6-sulfo-SLe^x, NeuAcα2-3Galβ1-4[Fucα1-3](6-O-sulfo)GlcNAc) supported detectable binding. Subsequent secondary screening was performed using surface plasmon resonance. Biotin glycosides immobilized on streptavidin biosensor chips were exposed to Siglec-8-Fc in solution. Whereas chips derivatized with SLe^x and 6-sulfo-SLe^x failed to support detectable Siglec-8 binding, 6'-sulfo-SLe^x supported significant binding with a K_D of 2.3 μM. In a separate test of binding specificity, aminopropylglycosides were covalently immobilized at different concentrations on activated (N-hydroxysuccinimidyl) glass surfaces (Schott-Nexterion Slide H). Subsequent exposure to Siglec-8-Fc precomplexed with FITC-anti-human Fc resulted in fluorescent signals at immobilized concentrations of 6'-sulfo-SLe^x as low as 5 pmol/spot. In contrast, SLe^x and 6-sulfo-SLe^x did not support any Siglec-8 binding at the highest concentration tested (300 pmol/spot). We conclude that Siglec-8 is a highly-specific lectin, binding preferentially to the SLe^x structure bearing an additional sulfate ester on the galactose 6-hydroxyl.

Resources and collaborative expertise were provided, in part, by The Consortium for Functional Glycomics, funded by grant GM62116 from the NIGMS, NIH. This work was funded by NIH grant AI41472 (to BSB)

(304) **Cell Signaling by Glycosylphosphatidylinositol Anchors of Plasmodium Falciparum**

Gowdahalli Krishnegowda, Jianzhong Zhu and D. Channe Gowda

Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, PA 17033.

Malaria caused by the protozoan parasite, *Plasmodium falciparum*, is a major public health problem around the world with a death toll of 1-2 million yearly. The process of malaria pathogenesis is highly complex and the underlying molecular mechanism remains poorly understood. Accumulated evidence suggests that the excessive production of proinflammatory cytokines and nitric oxide in response to acute parasitic infection contributes to the fever and development of cerebral and other organ-related severe malaria syndromes. Although comprehensive studies for determining various *P. falciparum* components that potentially contribute to the disease pathogenesis are lacking, the glycosylphosphatidylinositol (GPI) glycolipids are believed to be the prominent factors responsible for severe malaria. The deleterious effects of parasite GPIs have been attributed to their ability to produce proinflammatory cytokines and nitric oxide at elevated levels in deep vasculature of vital organs, where *P. falciparum*-infected erythrocytes generally sequester. This results in vascular and tissue damage and organ dysfunction, leading to severe pathological conditions. Understanding of cell signaling pathways involved in the induction of proinflammatory cytokines in response to the parasite GPIs is likely to offer therapeutic targets for malaria. Previous studies have shown that cell signaling by parasite GPIs involves the activation of PTK and PKC, which together regulate the activation of NF-*k*B/c-*rel* transcription factors with the downstream expression of proinflammatory responses. However, the details of the signaling pathway have not been studied previously. In the present study, we investigated the downstream signaling molecules involved in the production of proinflammatory cytokines, TNF-α, IL-12, IL-6, and nitric oxide in response to malarial GPIs. The data show that stimulation of mouse bone marrow-derived macrophages or human peripheral monocytes with *P. falciparum* GPIs leads to the activation of downstream MAPKs, ERK1/2, p38, JNK, and NF-*k*B. Further, studies using mice deficient in the activation of various MAPKs and specific inhibitors, demonstrate that the downstream MAPKs are differentially required for the NF-*k*B-dependent expression of various cytokines, chemokines, and nitric oxide. These results and those obtained from studies currently in progress will be presented. Supported by the grant AI41139 from NIAID, NIH.

(305) **Human EDEM2, a Novel Homolog of Family 47 Glycosidases, is Involved in ER-associated Degradation of Glycoproteins**

Steven W. Mast[1], Krista Diekman[2], Khanita Karaveg[1], Ann Davis[1], Richard N. Sifers [2] and Kelley W. Moremen[1]

[1] University of Georgia, Athens, GA 30602, [2] Baylor College of Medicine, Houston, TX 77030.

In the ER, misfolded proteins are retro-translocated to the cytosol and degraded by the proteasome in a process known as ER-associated degradation (ERAD). Early in this pathway, a proposed lumenal ER lectin, EDEM, recognizes misfolded glycoproteins in the ER, disengages the nascent molecules from the folding pathway, and facilitates their targeting for degradation. In humans there are a total of three EDEM homologs. The amino acid sequences of these proteins are different from other lectins, but are closely related to the Class I mannosidases (family 47 glycosidase). In this study, we have characterized one of the EDEM homologs from *H. sapiens*, which we have termed EDEM2 (C20orf31). Using recombinantly generated EDEM2, no alpha-1,2 mannosidase activity was observed. In HEK293 cells, recombinant EDEM2 is localized to the ER where it can associate with misfolded alpha 1-antitrypsin. Overexpression of EDEM2 accelerates the degradation of misfolded alpha 1-antitrypsin indicating that the protein is involved in ERAD.

(306) **Ganglioside Clustering Inhibits Axonal Regeneration by Activating a Rho/Rock Signaling Pathway**

Alka A. Vyas, Jennifer N. Stall, Susan E. Fromholt and Ronald L. Schnaar

Departments of Pharmacology and Neuroscience, The Johns Hopkins School of Medicine, Baltimore, MD 21205.

Gangliosides are sialylated glycosphingolipids distributed ubiquitously on vertebrate cell membranes, and are particularly abundant on nerve cells. Gangliosides have been proposed to reside in lipid rafts, dynamic and transient signaling platforms on cell membranes, where a variety of signaling components are segregated. This strategic location may enable them to mediate a variety of important physiological functions.

We and others have shown that gangliosides GD1a and GT1b function in maintaining axon-myelin stability and axonal cytoarchitecture, as well as in regulating axon regeneration. GD1a and GT1b serve as neuronal ligands for myelin associated glycoprotein (MAG). MAG (Siglec-4) is a member of the Siglec family of lectins and is one of the potent myelin inhibitors of axon regeneration. MAG has been shown to localize in myelin rafts and undergo oligomerization. Previously, we reported that clustering GD1a or GT1b on neuronal surfaces using multivalent complexes of highly specific IgG-class monoclonal antibodies inhibits axon outgrowth from cerebellar granule neurons in culture. Based on this and other observations, we proposed that

clustering of gangliosides GD1a and/or GT1b is responsible for MAG-mediated axon outgrowth inhibition. We now report our investigations of the signaling pathways leading from ganglioside clustering to axon outgrowth inhibition. Prior studies had implicated the p75 neurotrophin receptor, activation of the small GTPase RhoA, and activation of its downstream effector, ROCK (Rho-associated kinase) in myelin-mediated axon outgrowth inhibition. Consistent with this pathway, anti-GD1a and anti-GT1b antibody-mediated axon outgrowth inhibition from rat cerebellar granule cells was substantially reversed by a p75 antagonist peptide. The inhibitory effect of ganglioside antibody complexes was also reversed by adding a specific inhibitor of ROCK. Since these same inhibitors reverse inhibition by MAG, these data are consistent with the conclusion that MAG exerts it inhibitory effect by clustering GD1a and/or GT1b at the axonal or neuronal cell surface, leading to activation of p75, RhoA and ROCK.

This work was supported by NINDS grant NS37096. JNS was a Johns Hopkins University Summer Internship Program fellow from University of Notre Dame.

(307) **B Cell Signaling Pathways in CD22, ST6Gal I and CD22/ST6Gal I Null Mice**

Brian E. Collins[1], Per Bengtson[1], Lars Nitschke[2], Jamey D. Marth[3] and James C. Paulson[1]

[1] Departments of Molecular Biology and Experimental Medicine, The Scripps Research Institute, San Diego, CA, [2] Institute of Virology and Immunology, University of W¸rzburg, W¸rzburg, Germany, [3] The Howard Hughes Medical Institute and Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA.

CD22 (Siglec-2) is a co-receptor and negative regulator of B cell receptor signaling, and exhibits specificity for recognition of sialic acid containing ligands with the sequence Siaα2-6Gal. Mice deficient in CD22 (CD22 null) have been observed to exhibit a hyperimmune phenotype.[1] Conversely, mice deficient in ST6Gal I, and thus the ligand for CD22, are immuno-suppressed.[2] To determine if the phenotype of ST6Gal I null mice is indeed mediated through CD22, a double null mouse deficient in both CD22 and ST6Gal I has been generated. As reported by other laboratories, activation of B cells from CD22 null mice show slightly higher proliferation and higher Ca^{++} flux responses relative to WT, while B cells from the ligand deficient ST6Gal I null mice show significantly reduced responses in both assays. In contrast to the ST6Gal I single nulls, B cells from the CD22/ST6Gal I double null mice show levels of proliferation and calcium flux similar to those of wild type mice. These data suggest that the immuno-suppressed phenotype of the ST6Gal I null mice is mediated through CD22.

To further investigate the mechanism by which CD22 ligand interactions modulate B cell activation, we have begun to analyze the signaling pathways in these genetic models. By using phosphospecific antibodies, differences in phosphorylation of downstream signaling molecules are observed. Candidate molecules relevant to regulation by CD22 ligand interactions are being identified. These mice are anticipated to be a useful tool in identifying mechanisms of CD22 regulation of B cell receptor dependent and independent signaling pathways.

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(308) **Differential Regulation of Biosignals with GM1 Ganglioside in the Membrane Microdomains**

Koichi Furukawa[1], Masashi Nishio[1], Teruhiko Mitsuda[1], Satoshi Fukumoto[2] and Keiko Furukawa[1]

[1] Dep of Biochem II, Nagoya Univ Sch of Med, 65 Tsurumai, Showa-ku, Nagoya, Japan 466-0065

[2] Dep of Ped Dent, Nagasaki Univ Sch of Med, 1-7-1 Sakamoto, Nagasaki, Japan 852-8102.

Membrane microdomains such as caveolae, glycolipid-enriched microdomain (GEM) or detergent-insoluble microdomain (hereafter GEM/raft) are important in the regulation of various biosignals. To clarify the roles of acidic glycosphingolipids in the regulation of biosignals in GEM/raft, we studied the effects of over-expression of ganglioside GM1 in Swiss3T3 cells. In GM1+ Swiss3T3 cells, cell growth was clearly suppressed and phosphorylation levels of PDGF receptors (PDGFR) and ERK1/2 were also suppressed or rapidly reduced upon PDGF treatment. In the analysis of intra-cellular localization of PDGFR with sucrose density gradient fractionation of cell lysates, PDGFR was released from GEM/raft to non-GEM/raft fraction. To further analyze the roles of GM1 in the regulation of biosignals, transfectant cells of PC12 with GM1 synthase cDNA were analyzed for their responses to nerve growth factor (NGF) and epidermal growth factor (EGF). GM1-over-expressing transfectant cells (GM1+PC12) showed very poor neurite extension after NGF treatment, and phosphorylation and dimerization of NGF receptor TrkA were markedly reduced, although NGF binding was almost equivalent between the transfectant cells and controls. On the other hand, GM1+ PC12 showed rather increased proliferation with EGF treatment, particularly when cells were cultured in the medium with low serum concentration (2% or less). In the analysis of phosphorylation of EGF receptor (EGFR) and ERK1/2 upon EGF stimulation, it was shown that the phosphorylation levels of EGFR and ERK1/2 were sustained even after 60 min in GM1+PC12. To analyze the mechanisms for the differences in the effects of GM1-over-expression on the biosignals introduced with NGF and those with EGF, GEM/raft fractionation was performed. The distribution patterns of NGF receptors including TrkA and p75NTR markedly changed, i.e. they moved from GEM/raft to non-GEM/raft fraction with over-expression of GM1. This result resembled the behavior of PDGFR in Swiss3T3 with GM1 over-expression. In contrast, EGFR in PC12 was mainly localized in non-GEM/raft fraction before the transfection, and it showed minimal changes in the intra-cellular distribution after over-expression of GM1. Thus, receptors which originally exist in GEM/raft seem to easily undergo suppressive effects of GM1 expression due to the dispersion from GEM/raft fraction, resulting in the poor responses to the signals for differentiation/differentiation. On the other hand, receptors mainly localized in non-GEM/raft appear not to undergo marked influence with GM1 over-expression. The degree of GM1 binding with various receptors was examined with immunoprecipitation/TLC-immunostaining. The extracts from immunoprecipitates with anti-TrkA or anti-EGFR were served for TLC-immunostaining using cholera-toxin, showing marked differences in the binding of GM1, i.e. GM1 strongly binds with TrkA and weakly with EGFR. This might also explain the differential effects of GM1-over-expression on the two signal systems.

(309) **Kidney Lipids in Galcer Sulfotransferase-Deficient Mice: a Single Enzyme Catalyzes the Synthesis of Cell Membrane Barrier Sulfoglycolipids**

Keiko Tadano-Aritomi[1], Koichi Honke[2] and Ineo Ishizuka[1]

[1] Dept. Biochemistry, Teikyo Univ. Schl. Medicine, [2] Dept. Molecular Genetics, Kochi Med. Schl..

Sulfoglycolipids have been implicated in a variety of physiological functions based on their ion barrier activities and interaction with extracellular matrix proteins. They are specifically expressed in myelin sheath, spermatozoa, renal tubular cells, and epithelial cells of the gastrointestinal tract. The kidney is known to contain galactosylsulfatide, i.e. sulfated galactosylceramide (SM4s) as well as several sulfoglycolipids with longer carbohydrate chains and a sulfated ganglioside. Insight into the functions of these sulfoglycolipids has been provided by the upregulation of sulfoglycolipids of cultured renal cell lines in higher osmotic media. And then, the recent success in gene-targeted disruption of two enzymes which catalyze the synthesis of SM4s should decipher the mechanism of adaptation. The one is UDP-galactose:ceramide galactosyltransferase (CGT) which catalyzes the biosynthesis of GalCer, the precursor of SM4s. The other is 3'-phosphoadenylylsulfate-cerebroside (GalCer) 3'-sulfotransferase (CST), which sulfates GalCer to SM4s. Both CGT- and CST-deficient mice manifest neurological disorders caused by myelin dysfunction and male infertility due to the arrest of spermatogenesis. However, they did not show apparent morphological or physiological defects in the kidney. Instead, in the kidney of CGT-deficient mice, which lack both GalCer and SM4s, biosynthesis of more polar sulfoglycolipids, lactosyl sulfatide (SM3) and a bis-sulfated gangliotetraosylceramide (SB1a), was upregulated, suggesting that SM3 and SB1a can serve as surrogates for SM4s in the urine concentration process by renal tubules. The total lipid extract and the acidic lipid fractions prepared from the kidney of CST-deficient mice were analyzed by TLC and mass spectrometry. As expected, the homozygous mutant did not express SM4s. In addition, more polar sulfoglycolipids, SM3 and SB1a, were completely absent in the kidney. These data provide the first definitive evidence that the Cst gene is also responsible for the biosynthesis of SM3 and SB1a in vivo. In place of SM4s, its precursor GalCer, particularly that containing 2-hydroxy fatty acids (HFA) increased to the similar level of SM4s in the wild-type litter mates. Among major gangliosides in the kidney, the increase of GM1 up to approximately 10 times was outstanding in CST-deficient mice, while no significant difference was observed in GM3. The level of cholesterol 3-

sulfate did not change, indicating that the functions and/or histological distribution differ from those of sulfoglycolipids. Given that SB1a and GM1 share the same precursor glycolipids on the ganglio-series biosynthetic pathway, the increase in GM1 should be a compensatory process due to the lack of SB1a. Because the formidable adaptive capacity of the kidney to the alteration of osmotic environments is closely correlated with the metabolism of sulfoglycolipids, the increment in HFA-GalCer and/or GM1 could contribute to the normal kidney function in the CST-deficient mice.

(310) **Distribution of Galectin-1 in Adult Rat Dorsal Root Ganglia in vivo and in vitro**

Kazunori Sango[1], Akiko Tokashiki[1], Kyoko Ajiki[1], Hitoshi Kawano[1], Hidenori Horie[2] and Toshihiko Kadoya[3]

[1] Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu, Tokyo 183-8526, Japan, [2] Advanced Research Center for Biological Science, Waseda University, 2-7-5 Higashifushimi, NishiTokyo, Tokyo 202-0021, Japan, [3] Kirin Brewery Co. LTD, Takasaki, Gunma 370-0013, Japan.

Oxidized galectin-1 is a novel factor enhancing axonal regeneration in peripheral nerves after injury, but the process of extracellular release and oxidization of endogenous galectin-1 in the injured nervous tissue remains unknown. In this study, we examined the distribution of galectin-1 in adult rat dorsal root ganglia (DRG) in vivo and in vitro. By in situ hybridization histochemistry, galectin-1 mRNA was detected in all DRG neurons with a tendency for more intense signals to be observed in smaller neurons than in larger ones. Immunohistochemical analyses revealed that galectin-1 was distributed diffusely throughout the cytoplasm of smaller diameter neurons and Schwann cells in DRG sections. By double immunofluorescent staining, most of galectin-1-immunoreactive neurons expressed isolectin B4, but not calcitonin gene-related peptide (CGRP). This finding suggests that galectin-1 is expressed in glial cell line-derived neurotrophic factor (GDNF)-responsive neurons rather than nerve growth factor (NGF)-responsive neurons. Cytosolic galectin-1 in small DRG neurons may be involved in small fiber functions, such as nociception and thermoreception. In contrast to the studies in vivo, galectin-1 immunoreactivity was observed in almost all DRG neurons from an early stage (3 h) in culture and was restricted to the surface and/or extracellular region of neurons and Schwann cells at later stages (2, 4, and 7 days) in culture. Western blot analysis has revealed that both reduced and oxidized forms of galectin-1 were detected in culture media of DRG neurons and Schwann cells. These findings imply that galectin-1 is released from DRG neurons and Schwann cells upon axonal injury. Some of the molecules in the extracellular space may be converted to the oxidized form, which lacks lectin activity but could act on neural tissue as a cytokine-like molecule.

(311) **Dendritic Cells and the Recognition of Glycan Structures on Self and Non-Self Antigen**

Yvette van Kooyk, Irma van Die and Theo Geijtenbeek

Department of Molecular Celbiology and Immunology v.d. Boechorststraat 7, 1081 BT Amsterdam, the Netherlands.

Dendritic cells (DC) are specialized in the recognition of pathogens and play a pivotal role in the control of immunity. Yet DC are also important for homeostatic control recognizing self antigens and tolerizing its environment, indicating that the nature of the antigen it recognizes may steer a DC towards immunity or tolerance. C-type lectin receptors expressed by DC are involved in the recognition and capture glycosylated self antigens or pathogens. To date seven different C-type lectins have been identified on DC. It is now becoming clear that these C-type lectin receptors may not only serve as antigen receptor recognizing pathogens to allow internalisation and antigen presentation, but may also function in the recognition of self antigen, or as adhesion molecules and signaling molecules. We have studied in great detail the function and the glycan specificity of the DC-specific C-type lectin DC-SIGN. DC-SIGN recognizes high mannose structure and non-sialylated Lewis antigens (Lex, Ley, Leb and Lea) which are expressed on many pathogens, such as the envelope protein gp120 of HIV-1, and many other viral envelope glycoproteins, but also on the cell wall component ManLam of Mycobacteria. Targeting of these pathogens to DC-SIGN however leads to immune escape. These findings hint to a function of DC-SIGN in recognizing glycosylated self antigen to tolerize it environment. To date little is know on the specificity by which C-type lectins interact with self-glycoproteins. Lewis antigens are recognized on glycoproteins present on PMNs and mediate a cellular interaction between PMN and DC allowing proper antigen delivery. Also Lewis antigens on colon carcinomas are recognized by DC-SIGN on DC, and identification of the tumor antigens revealed that DC-SIGN strongly binds the tumor antigen CEA through Lex and Ley carbohydrate structures. Currently we are analyzing how glycan modifications in Lewis antigen during oncogenesis may suppress DC function in benefit for tumor growth. The finding that especially C-type lectins recognize carbohydrate structures on tumor cells opens up a new area of research that studies the potency of C-type lectins to interact with distinct glycosylated tumor antigens. Understanding the diversity of C-type lectins being expressed on DC as well as their carbohydrate specific recognition profile will be instrumental to understand DC pathogen recognition in many pathogenic disorders, as well as the regulation of cellular interactions of DC that are essential in the control of immunity.

(312) **Loss of Polysialic Acid Results in Serious Neurological Defects in Mice**

Birgit Weinhold[1], Herbert Hildebrandt[2], Ralph Seidenfaden[2], Martina M¸hlenhoff[1], Melanie Oschlies[1] and Rita Gerardy-Schahn[1]

[1] Abteilung Zellul‰re Chemie, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, 30625 Hannover, Germany, [2] Institut f¸r Zoologie, Universit‰t Hohenheim, Garbenstr. 30, 70593 Stuttgart, Germany.

Polysialic acid (polySia) is a unique and dynamically regulated posttranslational modification of the neural cell adhesion molecule NCAM. PolySia is the synthesis product of two polysialyltransferases, ST8SiaII and ST8SiaIV. In mice polysialylation of NCAM starts at embryonic day 9, is predominant in the perinatal phase and restricted to regions of neurogenesis, neuronal motility and plasticity in the adult animal. The essential role of polySia for learning and memory has been well documented.

Gene targeted mice exhibiting defects in either of the two polysialyltransferases are viable, fertile and show relatively mild phenotypes (1, 2). Here we present the first characterization of mice lacking both enzymes. ST8SiaII/ST8SiaIV double knockout mice are born apparently normal but then fail to thrive and display reduced viability compared to littermates carrying at least one functional polysialyltransferase allele. The double knockout mutants are completely devoid of polySia but express normal levels of NCAM in the brain. Prominent defects in these mice comprise smaller olfactory bulbs and disturbed neuroblast migration in the rostral migratory stream. The ST8SiaII/ST8SiaIV double knockout mice show dilated lateral ventricles progressing in some animals to a severe hydrocephalus with cortical thinning, corpus callosum hypoplasia and a deformed hippocampus as well as other neurological defects. The phenotype analysis of polySia-negative, NCAM-positive mice has been carried out in comparison to the existing NCAM knockout animals (lacking the polySia acceptor molecule NCAM). Detailed information highlighting unique and common characteristics of the two animal models will be given at the meeting.

1) Eckhardt M., Bukalo O., Chazal G., Wang L., Goridis C., Schachner M., Gerardy-Schahn R., Cremer H., Dityatev A.: J. Neurosci. 20: 5234-5244 (2000)

2) Angata K., Long J. M., Bukalo O., Lee W., Dityatev A., Wynshaw-Boris A., Schachner M., Fukuda M., Marth J. D.: J. Biol. Chem. published May 12 (2004) as doi:10.1074 [Epub ahead of print]

Supported by grants from the DFG, the Deutsche Krebshilfe and the Fonds der Chemischen Industrie

(313) **Identification of Glycoproteins with Embryoglycan in Embryonal Carcinoma Cells**

Hisako Muramatsu[1], Yoshihiro Oda[1] and Takashi Muramatsu[1,2]

[1] Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya, Japan,

[2] Department of Health Science, Faculty of Psychological and Physical Sciences, Aichi Gakuin University, Nisshin, Aichi, Japan.

The branched poly-N-acetyllactosamine with markers of early embryonic cells is abundantly expressed during early embryogenesis of mammals and is called embryoglycan. We attempted to isolate glycoproteins with embryoglycan based on the fact that Lotus tetragonolobus agglutinin □$B!J□(JLTA□$B!K□(J recognizes Lewis X structure in early embryonic cells and that Lewis X is largely on embryoglycan in these cells. Thus we applied detergent-extracted membrane glycoproteins from P19 embryonal carcinoma cells to a column of LTA-agrose. The bound glycoproteins were eluted by L-fucose, deglycosylated by N-glycanase, subjected to SDS-PAGE and digested with trypsin. The resulting peptides were separated by nano HPLC and analyzed by ion trap tandem mass spectrometry.

Consequently, we identified embigin and α6β1 integrin as LTA-binding glycoproteins. Embigin is a highly glycosylated molecule related to basigin, has two immunoglobulin domains and promotes integrin action. Embigin is strongly expressed in early embryos but its expression in the adult is restricted. We confirmed that α6β1 integrin has embryoglycan by analysis of the glycan produced in the presence of radioactive fucose. Previously we found that anti-Lewis X antibody inhibits cell-substratum adhesion of F9 embryonal carcinoma cells and that transfection with FUT4 increases cell-substratum adhesion of L cells. Taken together, we propose that Lewis X structure enhances integrin action by promoting interaction of integrins with other membrane glycoproteins such as embigin, and that Lewis X structure on embryoglycan exhibits stronger effects because of polyvalent ligands on it.

(314) **Expression of a Secreted Form of Dally, A *Drosophila* Glypican, Induces Overgrowth Phenotype by Affecting Action Range of Hedgehog**

Hiroshi Nakato[1], Satomi Takeo[2], Cyndy Firkus[1] and Takuya Akiyama[1]
[1] Department of Genetics, Cell Biology and Development, The University of Minnesota, Minneapolis, MN 55455, USA
[2] Department of Biology, Tokyo Metropolitan University, Hachioji-shi, Tokyo 192-0397, Japan.

Morphogens are signaling molecules that form a concentration gradient in a developmental field and can directly specify different cell fates in a concentration-dependent fashion.† In Drosophila wing development, three heparin-binding proteins act as morphogens and control pattern formation: Hedgehog (Hh), Decapentaplegic (Dpp), a bone morphogenetic protein-related protein, and Wingless (Wg), a Wnt family member.† In order to attain the precise gradient formation and its maintenance, the movement of these molecules must be tightly regulated.† However, molecular mechanisms for morphogen gradient formation are poorly understood.† Recent studies have suggested that glypicans, a family of heparan sulfate proteoglycans attached to the cell surface via a glycosylphosphatidylinositol-anchor, play essential roles in this process.† A Drosophila glypican, Dally, regulates gradient formation of Dpp in the developing wing.† † To gain insights into the function of glypicans in morphogen signaling, we constructed two mutant forms of Dally, a transmembrane form (TM-Dally) and a secreted form (Sec-Dally), and characterized their activities in tissue assembly as well as signal transduction.† Misexpression of tm-dally in the wing disc had a similar yet weaker effect in enhancing Dpp signaling compared to that of wild-type dally.† On the other hand, sec-dally expression led to severe defects in wing/notum formation, including ectopic formation and thickening of vein structures, blisters, wing folding, and induction of numerous ectopic notal bristles.† In addition to the patterning defects, sec-dally expression resulted in a substantial overgrowth of tissues and animals without enhancing the Dpp or insulin signaling pathways.† We found that all these abnormalities were caused by alterations of the action range of Hh. †Sec-dally expression in the wing discs expands the area of cells expressing Hh-target genes.† Normally, Hh protein is secreted from the posterior cells and forms a short-range gradient near the boundary of the anterior and posterior cells.† Anti-Hh antibody staining of sec-dally-expressing discs showed that Hh was abnormally distributed toward the anterior edge of the tissue.† Thus, Sec-Dally induces both patterning defects and overgrowth phenotypes by expanding the range of Hh protein distribution. † The alteration of the action range of Hh caused by sec-dally expression suggested a possible role of endogenous Dally in the Hh pathway. †Since Sec-Dally expressed only in the posterior (Hh-expressing) cells can affect Hh distribution, we asked if endogenous Dally has a role in these cells. †Co-immunoprecipitation experiments showed that Dally forms a complex with Hh.† In addition, overexpression of Dally sequesters Hh protein at the apical region of the wing disc.† We also found that dally mutation reduces levels of Hh protein in the producing cells, indicating that endogenous Dally is required to maintain normal levels of Hh.† These findings suggest that Dally is involved in the stabilization and/or intracellular trafficking of Hh protein in the producing cells.

(315) **Absence of Polysialic Acid Affect Neural Development in ST8Sia II and ST8Sia IV Double Knockout Mice.**

Kiyohiko Angata[1], Jamey D Marth[2] and Minoru Fukuda[1]
[1] Glycobiology Program, The Burnham Institute, La Jolla, CA 92037, [2] Howard Hughes Medical Institute, University of California San Diego, La Jolla, CA 92093.

The Neural cell adhesion molecule (NCAM) is a major glycoprotein in neural tissues and plays key roles in neurogenesis, cell migration, axonal pathfinding and neural plasticity. Polysialic acid, a linear homopolymer of α2,8-linked sialic acid, is mainly synthesized on NCAM and attenuate adhesive property of NCAM. Two polysialyltransferases, ST8Sia II (STX) and ST8Sia IV (PST), have been cloned and are capable of regulating NCAM function by controlling polysialic acid expression in cell-type specific and temporal manners. ST8Sia IV knockout mice exhibited deficiency in long-term memory of CA1 pyramidal cells through Schaffer-collaterals (1), while ST8Sia II knockout mice revealed polysialic acid function in mossy fiber projection and fear memory (2). In single mutant mice, polysialic acid remains in restricted and different cell types, suggesting complementary roles of each polysialyltransferase. Here, we generated double knockout mice to study *in vivo* roles of polysialic acid during neural development. The double knockout mice completely lost polysialic acid expression in the brains examined, demonstrating ST8Sia II and ST8Sia IV are responsible genes for polysialic acid synthesis in the brain. Mice without polysialic acid are smaller than other littermates and most of them die within 1 month, indicating indispensable role of polysialic acid. Lack of polysialic acid resulted in small olfactory bulb, enlarged lateral ventricle, thin cerebral cortex and immature cerebellum. In cerebrum, cells remain in anterior part of cortex and posterior cortex becomes as thin as a few cell layers. Accordingly, corpus callosum in posterior cortex is not fully developed in the double knockout mice, suggesting that both of neuronal and glial cell migration requires polysialic acid. In cerebellum, number of folia is decreased and alignment of Purkinje cells is disturbed in double knockout mice. Extension of axons and dendrites are also disturbed in double knockout mice. Neural cells labeled with BrdU during embryo migrate into cerebral and cerebellar cortices. In polysialic acid-deficient mice, migration of BrdU-labeled cells are less efficient than that of wild type mice in the same litter. Moreover, dead cells, probably due to apoptosis, were significantly increased in their brains. These results unveiled the roles of polysialic acid required for neural cell generation and migration during neural development, which are not found in NCAM-deficient mice. (Supported by NIH grant CA33895) 1. Eckhardt, M., Bukalo, O., Chazal, G., Wang, L., Goridis, C., Schachner, M., Gerardy-Schahn, R., Cremer, H., and Dityatev, A. (2000) J. Neurosci. 20: 5234-5244. 2. Angata, K., Long, J.M., Bukalo, O., Lee, W., Dityatev, A., Wynshaw-Boris, A., Schachner, M., Fukuda, M., and Marth, J.D. (2004) J. Biol. Chem. 279: 32603-32613.

(316) **Differentiation of F9 Embryonal Carcinoma Cells Requires Up-regulation of Gangliosides Biosynthesis and Formation of Membrane Microdomains**

Takashige Sato[1], Asif Mohammad Zakaria[1], Satoshi Uemura[1], Atsushi Ishii[3], Yoshiko Ohno-Iwashita[4], Yasuyuki Igarashi[1] and Jin-ichi Inokuchi[1,2]
[1] Department of Biomembrane and Biofunctional Chemistry, Hokkaido University, [2] Core Research for Evaluational Science and Technology program (CREST), Japan Science and Technology Corporation (JST), Graduate School of Pharmaceutical Sciences, Frontier Research Center for Post-Genomic Science and Technology, [3] Division of Project Research, Creative Research Initiative "Sousei" Hokkaido University, [4] Biomembrane Research Group, Tokyo Metropolitan Institute of Gerontology.

Mouse F9 embryonal carcinoma cells have been widely used as a model for studying the mechanism of embryonic differentiation, since they are similar to the inner cell mass of early mouse embryos and can differentiate into primitive endoderm (PrE) following retinoic acid (RA) treatment. During F9 cell differentiation, the carbohydrate chains of glycoproteins and their corresponding glycosyltransferases are known to undergo rapid changes. However, there have been no corresponding reports on the expression of gangliosides. Gangliosides, sialic acid-containing glycosphingolipids (GSLs), are found on the surface of the plasma membrane of all vertebrate cells and are involved in signal transduction, cell growth, and differentiation. We have developed a custom cDNA array that is highly sensitive for the genes responsible for sphingolipid biosynthesis and metabolism. Using this, we found that, of the 28 selected genes, 26 exhibited increased expression during F9 differentiation into PrE. Neutral glycosphingolipids were expressed, at similar levels before and after differentiation, however in undifferentiated F9 cells, the expression of GM3 synthase was low, and there were no detectable signals for GD3 synthase or GQ1b synthase, correctly predicting an absence of b-series gangliosides. On the other hand, the differentiated F9 cells expressed genes responsible the

biosynthesis for a- and b-series gangliosides at high levels. The information from the SL array was supported by TLC analyses of GSLs, which exhibited an approximately 24-fold increase in total gangliosides during the differentiation. Ganglioside depletion by glucosylceramide synthase inhibitors, (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) and its analog) resulted in delayed expression of Disabled-2 suggesting the involvement of gangliosides in F9 cell differentiation. Disruption of cholesterol-enriched membrane microdomains by methyl-b-cyclodextrin (MβCD) also delayed differentiation. Both MβCD and D-PDMP blocked the accumulation of src family kinases (SFKs) to microdomains. However, D-PDMP did not block flotillin accumulation, yet MβCD did. These results suggest that SFKs interact with gangliosides. Therefore, we investigated the interaction between SFKs and gangliosides and we could demonstrate here that the direct association between gangliosides and SFKs by co-immunoprecipitation. Additionally, confocal laser microscopy revealed the formation of distinct functional microdomains integrating SFKs with gangliosides and cholesterol during PrE differentiation. Thus, we demonstrate, for the first time, the outstanding upregulation of ganglioside biosynthesis and its importance in the formation of distinct microdomains incorporating SFKs with ganglioside during RA-induced differentiation of F9 cells. We think that a comprehensive analysis of proteins that are selectively translocated into microdomains in response to ganglioside increases during differentiation, would be a useful strategy in revealing the functional roles of gangliosides in microdomains.

(317) Down Regulation of Trypsinogens Expression In α1,6-Fucosyltransferase-Deficient Mice

Wenzhe Li[1,4], Takatoshi Nakagawa[1,5], Nobuto Koyama[3,5], Jianguo Gu[2,5], Eiji Miyoshi[2,5], Naoyuki Taniguchi[2,4] and Akihiro Kondo[1,4,5]

[1] Department of Glycotherapeutics, , [2] Department of Biochemistry, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka, 565-0871, Japan, [3] Takara Bio Inc. 3-4-1 Seta, Otsu, Shiga, 520-2193, Japan, [4] The 21st century COE program, [5] CREST.

Alpha 1,6-fucosyltransferase (FUT8) catalyzes the transfer of fucose into α1,6-linkage to the innermost GlcNAc of N-glycans (core fucosylation). The core fucosylated N-glycans are widely distributed in mammalian tissues. It was reported that the core fucosylation was related to the increased efficacy of antibody dependent cellular cytotoxicity (ADCC) activity of the IgG. Recently, we developed FUT8 knockout ($FUT8^{-/-}$) mouse, and found that the $FUT8^{-/-}$ mice showed growth retardation and earlier postnatal death. In order to investigate underlying molecular mechanism, we examined mRNA expression levels using IntelliGene II mouse DNA Chip containing almost 5,000 genes. We compared mRNA expression profiles of wild type (18 days gestation) versus $FUT8^{-/-}$ (18 days gestation) embryos. We found that 37 genes showed more than 2-fold changes in their expressions between both embryos. Among these genes, 23 genes were up-regulated, and 14 genes were down-regulated in $FUT8^{-/-}$ mice. Particularly, four trypsinogen mRNAs on this DNA chip were dramatically suppressed. Consistent with microarray data, the expression levels of trypsinogen mRNAs and proteins in the duodenum, small intestine and pancreas were confirmed by real-time RT-PCR and Western blotting, respectively. Gelatin zymography analysis also confirmed the presence of trypsin, an active form of trypsinogen in duodenum, small intestine and pancreas. To further investigate the meanings of trypsinogen suppression, we established FUT8 knockdown cell lines using pancreatic acinar cell line (TGP49) and intestinal epithelial cell line (MODE-K) by a stable RNA interference (RNAi) technique. As expected, the expression of trypsinogens was markedly inhibited in FUT8 knockdown cell lines. These studies could provide new insights into FUT8 function.

(318) Neuronal expression of the chondroitin sulfate proteoglycans RPTPβ and phosphacan

Noriko Hayashi[1], Seiji Miyata[1], Mayumi Yamada[1], Kaeko Kamei[1] and Atsuhiko Oohira[2,3]

[1] Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan, [2] Department of Perinatology and Neuroglycoscience, Institute for Developmental Research, Aichi Prefectural Colony, Kasugai, Aichi 480-0392, Japan, [3] Graduate School of Medicine, Nagoya University, Nagoya 466-8550, Japan.

Receptor-type protein-tyrosine phosphatase β (RPTPβ) and its spliced variant phosphacan are major components of chondroitin sulfate proteoglycans in the central nervous system. In this study, expression and localization of RPTPβ and phosphacan were examined in developing neurons by immunological analyses using 6B4, 3F8, and anti-protein-tyrosine phosphatase (PTP) antibodies and reverse transcription-polymerase chain reaction (RT-PCR). Light microscopic immunohistochemistry showed that 6B4 RPTPβ/phosphacan immunoreactivity was observed around neurons in the cortical plate. Further ultrastructural observation showed that 6B4 RPTPβ/phosphacan immunoreactivity was observed mainly at membrane of migrating neurons and radial glia. Immunocytochemical analysis revealed that RPTPβ immunoreactivity was observed in cultured cerebral, hippocampal, and cerebellar neurons in addition to type-1 and type-2 astrocytes. Western analysis further demonstrated that shorter receptor form RPTPβ (sRPTPβ) was detected from cell lysate of cortical and hippocampal neurons using 6B4 and anti-PTP antibodies, while sRPTPβ of cerebellar neurons and type-1 astrocytes was recognized only by anti-PTP antibody. Phosphacan was detected from neuronal culture supernatants of cortical, hippocampal, and cerebellar neurons, but not from type-1 astrocytes using 6B4 and 3F8 antibodies. RT-PCR analysis demonstrated the prominent expression of sRPTPβ and phosphacan mRNAs in cortical neurons, and that of phosphacan mRNA in type-1 astrocytes. During culture development of cortical neurons, the immunoreactivity of 6B4 sRPTPβ was observed entirely on neuronal surface including somata, dendrites, axons, and growth cones at earlier stages of cortical neuronal culture such as stage 2 and 3, while, after longer culture, 6B4 sRPTPβ immunoreactivity in stage 4 and 5 neurons was detected at dendrites and somata and disappeared from axons, and never observed over axonal terminals and postsynaptic spines. These results demonstrate that neurons are able to express sRPTPβ on their cellular surface and to secrete phosphacan, and neuronal expression of sRPTPβ may modulate neuronal differentiation including neuritogenesis and synaptogenesis.

(319) Significance of Glycosphingolipid Synthesis in Zebrafish Embryogenesis

Mitsuyoshi Masuda[1], Noriyuki Sueyoshi[1], Sayaka Satoh[1], Yukihiro Yoshimua[1], Keishi Sakaguchi[1], Nozomu Okino[1], Yoshio Hirabayashi[2] and Makoto Ito[1]

[1] Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka, Japan, [2] RIKEN, Saitama, Japan.

Glycosphingolipids (GSLs), present in the lipid microdomains of vertebrates, are involved in a variety of important biological processes. GSLs are also known to be receptors for pathogens and their toxins. GSLs are synthesized by step-by-step additions of monosaccharides from the corresponding nucleotide sugars, which are catalyzed by specific glycosyltransferases. The first step of glycosylation of ceramide is usually initiated with the transfer of glucose to ceramide, which is catalyzed by UDP-glucose:ceramide glucosyltransferase-1 (GlcT-1, GlcCer synthase). Based on the nucleotide sequence of the human GlcT-1 gene, we isolated a cDNA clone encoding zebrafish GlcT-1 (zGlcT-1), which possessed an open reading frame of 1,179 base pairs encoding 393 amino acids. During embryogenesis, the activity of zGlcT-1 increased transiently at 9 hours postfertilization (9 hpf, 90%-epibody stage), then decreased to the initial level at 24 hpf, and again gradually increased by 72 hpf, at which time the activity was 40-fold higher than that of 1-cell-stage embryos. The knockdown of the zGlcT-1 gene with morpholino-based antisense oligonucleotides (AMO) led to an increase in embryos with severe morphological and cellular abnormalities, i.e., a significant increase in round-shaped embryos and an increase in apoptotic cells in the midbrain and hindbrain. A marked loss of zGlcT-1 activity and GSLs was found in the AMO- but not in the mismatch oligo-injected embryos. Unexpectedly, however, no increase in ceramide content was observed by the knockdown of zGlcT-1, suggesting that the abnormal phenotype was not due to the accumulation of ceramide during embryogenesis. Interestingly, the simultaneous injection of glucosylceramide with the AMO partially rescued the phenotype and recovered the GSL content of embryos. These results clearly show the significance of zGlcT-1 and GSL synthesis in zebrafish embryogenesis.

(320) Gene Expression Profiling of Mouse Postnatal Cerebellar Development Using Oligonucleotide Microarrays Designed to Detect Differences in Glycoconjugate Expression

Frances I. Smith[1], Qiang Qu[1], Tim Gilmartin[2] and Steven Head[2]

[1] University of Massachusetts Medical School, Shriver Center, 200 Trapelo Road, Waltham. MA. 02452, [2] The Scripps Research Institute, DNA Array Core Facility, 10550 N Torrey Pines Rd, La Jolla CA, 92037.

Differences in gene expression patterns from adult and postnatal day 7 (P7) mouse cerebellum RNA were analyzed by hybridization to the GLYCOv2 glycogene array, a custom designed oligonucleotide array focused on glycosyl transferases, carbohydrate binding proteins, proteoglycans and related genes. Two methods were used in the analysis: One in which replicate probeset copies were averaged and one in which each probeset copy was treated independently. 171 genes were identified by both methods as being differentially expressed with statistical confidence. 41 additional genes were identified using the independent probe copy method and 2 additional genes were identified using the averaging method. Within this group, sialyltransferases (SiaTs) that modify glycoproteins were elevated at P7, whilst SiaTs that preferentially modify glycolipids were elevated in the adult, consistent with a role for gangliosides in maintaining neuronal function in the adult. Also within this group, three proteoglycans- versican, bamacan and glypican- were elevated at P7, along with growth factor midkine, which is known to bind to multiple types of proteoglycans, and fibroblast growth factor receptor 1, whose activity is known to be influenced by heparin sulfate proteoglycans. Three sulfo-transferases that modify the extent of proteoglycan sulfation were also differentially regulated, and may modify the interaction of the proteoglycans with their binding partners during cerebellar development.

(321) **Small Changes in Immune Cell Development and Function as a Result of Tissue Specific Disruption of Heparan Sulfate**

Omai B. Garner[1], Vibeke Videm[2] and Jeffrey D. Esko[1]

[1] Department of Cellular Molecular Medicine, Glycobiology Research and Training Program, University of California, San Diego, La Jolla, California, 92093-0687, [2] Department of Immunology and Transfusion Medicine, Trondheim University Hospital, Trondheim N-7006, Norway.

The development and function of the adaptive arm of the immune system (B and T cells) depends upon critical interactions with cytokines, cell-cell contact, and cell-extracellular matrix interplay. All of these processes are thought to depend on heparan sulfate, but the functional significance of heparan sulfate has not been elucidated in vivo. To address this question, we altered the structure of heparan sulfate in B and T cells by tissue specific deletion of heparan sulfate GlcNAc N-deacetylase/N-sulfotransferase (NDST 1 and 2) in T cells, and the copolymerase EXT-1 in B cells. T cells showed no change in overall number of lymphocytes in each lymphatic compartment, but the spleen exhibited alteration in follicle size and organization. T cells with altered heparan sulfate also exhibited a functional hypersensitivity to activation by CD3 mAb, but the effect was small in comparison to other changes in N-linked glycosylation (e.g., Mgat5 deficiency). B cells showed minor developmental changes in response to altering heparan sulfate. They also showed small changes in production of antibody in response to in vivo stimulation. Together, these findings indicate that heparan sulfate on T and B cells play a minor role in lymphocyte development and function. Thus, some of the effects commonly attributed to heparan sulfate in the immune system may be more related to glycosaminoglycans in the surrounding matrix rather than those produced by lymphocytes.

(322) **Defective Mammary Development in Mice Containing a Tissue-Specific Deletion of a Heparan Sulfate Sulfotransferase**

Brett E. Crawford , Omai B. Garner, Jan Castagnola and Jeffrey D. Esko

Department of Cellular and Molecular Medicine, Glycobiology Research and Training Program, University of California, San Diego, La Jolla, California, 92093-0687.

The development of the mammary gland is thought to depend on the sequential action of several heparin binding growth factors. However, the in vivo significance of the interaction of these factors with heparan sulfate not been elucidated. To address this problem, we have examined the role of heparan sulfate in mammary gland development by tissue-specific deletion of heparan sulfate GlcNAc N-deacetylase/N-sulfotransferase-1 (NDST1) in wildtype and NDST2 null mice. NDST1 and 2 are enzymes that are critical for initiating the modification of heparan sulfate. Mammary epithelia lacking both isozymes fail to undergo branching morphogenesis, whereas epithelia lacking only NDST1 develop normally to sexual maturity but fail to lactate. The epithelia of NDST1 deficient glands are highly apoptotic at day 1 of lactation, exhibiting reduced phosphorylation of AKT/PKB and decreased cyclin D1 expression. In vitro experiments suggested that undersulfation of heparan sulfate disrupted heregulin signaling through ErbB receptors. These findings show that NDST1 or NDST2 modified heparan sulfate is sufficient for branching morphogenesis in the mammary gland; however, NDST1 is required for lobuloalveolar development. We attribute these differences to unique modifications of heparan sulfate generated by each of these two enzymes.

(323) **Core 2 beta 1-6 N-acetylglucosaminyltransferase-III selectively contributes to P-selectin ligand formation in activated CD8 T cells**

Jasmeen S. Merzaban, Jonathon Zuccolo, StÈphane Y. Corbel, Michael J. Williams and Hermann J. Ziltener

The Biomedical Research Centre, University of British Columbia, 2222 Health Sciences Mall, Vancouver BC V6T 1Z3 Canada.

The core 2 beta 1-6 N-acetylglucosaminyltransferase (C2GlcNAcT) family of enzymes synthesize O-glycan branches. C2GlcNAcT-I gene-deletion studies have demonstrated an essential role for this enzyme in the control of selectin ligand mediated cell trafficking, while little is known about the role of the two alternate core 2 isoenzymes: C2GlcNAcT-II and C2GlcNAcT-III. We have examined activated splenocytes from C2GlcNAcT-I^{null} mice for their ability to express P-selectin ligands (P-selL) and have found that C2GlcNAcT-I independent P-selL formation can occur in CD8 T cells, but not in CD4 T cells, activated under high density cell culture conditions. Cell surface binding of P-selectin could be inhibited with the neutralizing anti-PSGL-1 antibody, 2PH-1, confirming PSGL-1 as the P-selectin ligand. CD8 T cells from C2GlcNAcT-I^{null} were capable of rolling under shear flow on immobilized P-selectin. Real time RT-PCR analysis identified significant levels of C2GlcNAcT-III, but not C2GlcNAcT-II, RNA in activated splenocytes, identifying the C2GlcNAcT-III enzyme as the likely source of core 2 activity. Up-regulation of P-selL correlated with an increase in core 2 enzyme activity measured by a standard enzymatic assay and by cell surface binding of the core 2 sensitive mAb 1B11, revealing the well established C2GlcNAcT-I substrates CD43 and CD45 as further physiological targets of C2GlcNAcT-III. To assess whether C2GlcNAcT-I independent P-selL induction occurs under in vivo conditions, we analyzed T cells from mice transgenic for the male antigen (HY) T cell receptor. CFSE labeled HY^{tg} CD8 T cells from female C2GlcNAcT-I^{null} or wild type donors were transferred into male recipient mice. The CD8 T cell response along with P-selL induction was measured 2-4 days after cell transfer. Both wt and C2GlcNAcT-I^{null} CD8 T cells showed a comparable proliferative response. While wt controls expressed high levels of P-selL, C2GlcNAcT-I^{null} cells also expressed P-selL, although at reduced levels. Our data thus indicate that C2GlcNAcT-III may contribute to P-selL formation and cooperate with C2GlcNAcT-I in the control of CD8 T cell trafficking.

(324) **Roles of Extended Core 1 *O*-Glycans in Lymphocyte Trafficking: Analysis of Beta1,3-*N*-Acetylglucosaminyltransferase-3 Knockout Mice**

Junya Mitoma, Hiroto Kawashima and Minoru Fukuda

Glycobiology Program, Cancer Research Center, The Burnham Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037.

The interaction of L-selectin on lymphocyte and L-selectin ligand expressed on high endothelial venules (HEV) of peripheral lymph nodes (PLN) leads to the lymphocyte rolling and eventual lymphocyte extravastion. HEV cells express several sialomucins such as CD34 and GlyCAM-1 which are modified with 6-sulfo sialyl Lewis x structure, a functional L-selectin ligand. In mucin-type *O*-glycans, 6-sulfo sialyl Lewis x can be added to core 2 branch and extended core 1 structures. Core 2 branch is synthesized by core 2 beta1,6-*N*-acetylglucosaminyltransferase-I (Core2GnT-I), and Core2GnT-I knockout mice showed ~ 50% decrease of lymphocyte homing (1). The synthesis of extended core 1 structure requires beta1,3-*N*-acetylglucosaminyltransferase-3 (beta3GlcNAcT-3) which is also called Core1-beta1,3-*N*-acetylglucosaminyltransferase (2), one of the seven beta1,3-*N*-acetylglucosaminyltransferases reported so far. 6-sulfo sialyl Lewis x on extended core 1 *O*-glycans is recognized by MECA-79 antibody (2) which completely inhibits lymphocyte homing into PLN. We have also demonstrated that beta3GlcNAcT-3 is the only beta3GlcNAcT which reconstitutes MECA-79 antigen when cultured cells are transfected with L-selectin ligand sulfotransferase (3).

To determine the significance of the extended core 1 *O*-glycans in lymphocyte homing, we generated beta3GlcNAcT-3 knockout mice. In these mice, beta3GlcNAcT-3 gene was replaced with cDNA encoding enhanced green fluorescent protein (EGFP) to monitor the expression of beta3GlcNAcT-3 *in vivo*. beta3GlcNAcT-3 (-/-) mice developed normally and lacked any obvious gross anomaly. As expected, EGFP expression was observed in HEV of lymph nodes. Significantly, MECA-79 reactivity was completely absent in HEV of beta3GlcNAcT-3 (-/-) mice, demonstrating that beta3GlcNAcT-3 is the only beta3GlcNAcT which can add GlcNAc to

core 1 *O*-glycan in beta1,3-linkage in HEV. The binding of L-selectin-IgM chimera to HEV was appreciably reduced, and in Stamper-Woodruff assay lymphocytes failed to bind to PLN prepared from beta3GlcNAcT-3 (-/-) mice *in vitro*. Moreover, lymphocyte homing into PLN was also decreased to ~ 60% of wild-type mice. These results indicate that the formation of extended core 1 structure participates in the synthesis of functional L-selectin ligand in PLN in addition to core 2 branch. To determine the contribution of core 2 and extended core 1 *O*-glycans in lymphocyte homing, we are now studying mice deficient in both Core2GnT-1 and beta3GlcNAcT-3. Supported by NIH grants CA48737 and CA71932.

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(2) Yeh et al. Cell **105**: 957-969 (2001)
(3) Mitoma et al J. Biol. Chem. **278**: 9953-9961 (2003)

(325) **Enzymatic Activity of α-mannosidase IIx in *N*-glycan biosynthesis**

Tomoya O Akama[1], Jun Nakayama[2], Hiroaki Nakagawa[3], Nyet Wong[4], Mark Sutton-Smith[4], Shin-ichiro Nishimura[3], Kelley W Moremen[5], Anne Dell[4], Jamey D Marth[6] and Michiko N Fukuda[1]
[1] The Burnham Institute, La Jolla, CA, [2] Shinshu University, Matsumoto, Japan, [3] Hokkaido University, Sapporo, Japan, [4] Imperial Collage, London, UK, [5] University of Georgia, Athens, GA, [6] University of California San Diego, La Jolla, CA.

α-mannosidase IIx (MX) is an enzyme that has high homology with an *N*-glycan processing enzyme, α-mannosidase II (MII) (1). Human MX protein showed α-mannosidase activity over synthetic substrate (1), and overexpression of human MX in CHO cells changed profile of *N*-glycans produced in the cells (2). Moreover, MX-deficient male mice showed infertile phenotype that was primarily caused by a lack of specific *N*-glycan structure on male germ cells (3). These results suggested that MX is another α-mannosidase in *N*-glycan processing. However, the role of MX in *N*-glycan biosynthesis *in vivo* has not been unambiguously established yet. To elucidate the role of MX in *N*-glycan biosynthesis *in vivo*, we produced MII/MX double knock-out (DKO) mice. Most of MII/MX DKO mice died soon after birth because of respiratory failure. Histological and electron microscopic analyses revealed abnormalities in the lung, liver and kidney tissues of DKO, suggesting that inactivation of both MII and MX enzymes leads significant damages to these tissues, while either MII-deficient or MX-deficient mutants did not show a significant defect in these tissues. No morphological abnormalities were evident in DKO in other tissues such as intestine, suggesting that morphologically visible abnormalities in MII/MX DKO are tissue-type specific. We analyzed *N*-glycan structures from DKO embryos by lectin histochemistry, lectin blot, two-dimensional HPLC and mass spectrometry. These analyses detected no complex type *N*-glycans in DKO. Both HPLC analysis and mass spectrometry revealed a prominent accumulation of hybrid-type structures with five mannose residues in DKO, indicating the arrest of *N*-glycan processing at the step of MII. Although the fibroblasts prepared from DKO embryo were not stained by E-PHA lectin, these cells restore complex-type carbohydrate production after they were transfected with an expression vector for MX. These results clearly indicate that MX is an integral part of *N*-glycan processing *in vivo* in the mouse, and is responsible for the alternative pathway, which has been proposed by the study of MII gene KO mouse (4).

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(2) Oh-eda, M. et al., Eur. J. Biochem., 268, 1280-1288 (2001)
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(4) Chui, D. et al., Cell, 90, 157-167 (1997)

(326) **Involvement of Galnac 4-Sulfate 6-*O*-Sulfotransferase in the Synthesis of Chondroitin Sulfate E in Bone Marrow Derived Mast Cells**

Shiori Ohtake[1,2], Toshiko Morisaki[1], Sachiko Kondo[1], Kaori Matsumura[1], Koji Kimata[2] and Osami Habuchi[1]
[1] Department of Chemistry, Aichi University of Education, Igaya-cho, Kariya, Aichi 448-8542, Japan , [2] Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-1195, Japan.

Bone marrow derived mast cells (BMMC) synthesizing chondroitin sulfate E (CS-E) as a granular component have been studied as a model of mucosal mast cells. We previously identified cDNA of human GalNAc 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST) and found that recombinant GalNAc4S-6ST is able to synthesize CS-E in vitro. We examined whether GalNAc4S-6ST expressed in BMMC is involved in the synthesis of CS-E, and obtained following results: (1) cellular extracts from BMMC showed a sulfotransferase activity resembling to GalNAc4S-6ST, (2) expression of GalNAc4S-6ST as well as C4ST was increased during the development to BMMC, (3) the specificity of recombinant mouse GalNAc4S-6ST prepared from RNA expressed in BMMC was almost the same as the specificity of the sulfotransferse contained in the cellular extracts from BMMC, and (4) the proportion of the nonreducing terminal sulfation in vitro by GalNAc4S-6ST was increased when the pH of the reaction mixture was elevated, whereas the proportion of the synthesis of the nonreducing terminal GalNAc(4,6-SO_4) residue of chondroitin sulfate in BMMC was increased when BMMC were cultured in the presence of bafilomycin A. In addition, we found that the treatment of BMMC with bafilomycin A resulted in the marked decrease in 6-sulfation of the GalNAc(4SO_4) residues despite of a minimal decrease in 4-sulfation of GalNAc residues. These observations strongly suggest that GalNAc4S-6ST expressed in BMMC is present in the compartments with rather low pH and should be involved in the synthesis of CS-E, and that the compartment synthesizing CS-E may be different from the compartment where 4-sulfation of GalNAc residues occurs.

(327) **The Functional Analysis of *Drosophila* Protein *O*-Mannosyltransferases Using Rnai Mutant Flies**

Tomomi Ichimiya[1,2], Hiroshi Manya[3], Yoshiko Ohmae[1], Hideki Yoshida[1,2], Ryu Ueda[2,4], Tamao Endo[3] and Shoko Nishihara[1,2]
*[1] Laboratory of Cell Biology, Department of Bioinformatics, Faculty of Engineering, Soka University,
[2] Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Agency (JST),
[3] Glycobiology Research Group, Tokyo metropolitan Institute of Gerontology, Foundation for Research on Aging and Promotion of Human Welfare, [4] Invertebrate Genetics Laboratory, National Institute of Genetics.*

O-mannosylation is an important modification of proteins in various fundamental physiological processes. The type of muscular dystrophy like Walker-Warburg syndrome and muscle-eye-brain disease was caused by *O*-link glycosylation defects of α-dystroglycan. These defects were involved in mutation of protein *O*-mannosyltransferase (POMT) and protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase (POMGnT). *Drosophila melanogaster* has two protein *O*-mannosyltransferase, *dPOMT1* and *dPOMT2*, which correspond to human *hPOMT1* and *hPOMT2*, respectively. To obtain information about the function of dPOMT1 and dPOMT2 *in vivo*, we tried to make RNAi mutant flies using the *GAL4-UAS-IR* system. Both of the RNAi mutant flies showed the same *rt* phenotypes, in which the abdomen is twisted through 30° to 60°, as classical *dPOMT1* mutants. The genetic interaction analysis revealed a synergistic effect between these two mutations, suggesting that the two gene-products function in the same genetic cascade. The result of whole mount *in situ* hybridization showed that *dPOMT1* and *dPOMT2* were coexpressed. We also performed biochemical analyses to demonstrate that recombinant dPOMTs function as a protein *O*-mannosyltransferase. Whereas there was no enzyme activity when either dPOMT1 or dPOMT2 was expressed independently, there appeared POMT activity when dPOMT1 and dPOMT2 were coexpressed. These results demonstrated that coexpression of dPOMT1 and dPOMT2 was an essential factor for revealing their activities. In each RNAi mutant fly, POMT activity was also reduced. It supports the heterophilic nature of these two enzymes. These described data indicate that both *Drosophila* POMT1 and POMT2 are required for functional POMT activity to contribute to normal muscle development *in vivo*. To know the existence of extended *O*-mannosylglycans in *Drosophila*, we tried to search *Drosophila* homologs of the *hPOMGnT1* gene, but could not obtain any *Drosophila* ones. Accordingly, we measured POMGnT activity of two *Drosophila* mannose β1,2-*N*-acetylglucosaminyltransferases, dMGAT1 and dMGAT2, as candidates for POMGnT. There was no POMGnT activity in the recombinant dMGAT1 and dMGAT2 protein. These results imply that the extended *O*-mannosylglycans, Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-Ser/Thr, found in humans is absent in *Drosophila* because there is no POMGnT. Considering the above results, a single mannosyl modification might be enough in *Drosophila*, while extended *O*-mannosylglycans are integrant in humans.

(328) **A New Slit Receptor with Galactose-Binding Lectin Domains has a Role in Axon Guidance in C. Elegans**

Joseph G. Culotti[1,2] and Kazuko Fujisawa[1]
[1] S.L. Research Institute, Mt Sinai Hospital, Toronto, Canada, [2] Depart,ment of Molecular and Medical Genetics.

The highly glycosylated, dorsally expressed extracellular SLT-1/Slit guidance cue protein acts through the SAX-3 receptor, in parallel with the ventral attractant UNC-6/Netrin, to guide growing axons toward the ventral

nerve cord of C. elegans (Hao et al.,Neuron 32:25-38, 2001). Here we identify an evolutionarily conserved gene, eva-1(ev751), that functions in the same pathway as SLT-1/Slit and SAX-3/Robo to guide axons projecting from the lateral cell body of the AVM and PVM sensory touch neuron to targets in the ventral nerve cord. Expression data and phenotype characterization of mutants and transgenic lines suggest that EVA-1 is required for the SLT-1 dependent functions of SAX-3, that EVA-1 provides cell specificity to SLT-1 and SAX-3 dependent guidance mechanisms and that EVA-1 is a novel receptor for SLT-1. The extracellular domain of EVA-1 has two predicted galactose-binding lectin domains (aka SUEL domains) found in other nervous system proteins, like proteins that bind α-latrotoxin. These domains appear essential for EVA-1 function. Our data also indicate that at 25oC EVA-1 and SLT-1 normally prevent SAX-3 from inhibiting an unknown ventral guidance mechanism (possibly UNC-6/netrin signaling) in the AVM neuron.

(329) **Development of Sensitive Chemical and Immunochemical Methods for Detecting Sulfated Sialic Acids and their Application to Localization and Quantitation Studies of Sea Urchin Sperm and Eggs**

Nao Yamakawa[1,2], Eri Maehashi[1], Shinji Miyata[1,2], Chihiro Sato[1], Kimio Furuhata[3] and Ken Kitajima[1,2,4]

[1] Graduate School of Bioagricultural Sciences, Nagoya University, [2] Bioscience and Biotechnology Center, Nagoya University, [3] School of Pharmaceutical Science, Kitasato University , [4] Institute for Advanced Research, Nagoya University.

Sialic acids (Sia) are widely distributed in glycoconjugates such as glycoproteins and glycolipids, and play important biological roles. Sia are often modified by various substituents on the carbons 4, 5, 7, 8, and 9, generating a family of more than 50 different members. The expression of these modified Sia is tissue-specific and developmentally regulated. However, only a few reports demonstrating the biological significance of these modified Sia have been published to date. Sulfation is a unique modification of Sia, in that it introduces an additional negative charge to Sia. Sulfated Sia residues have been demonstrated to occur in sea urchin sperm and egg and bovine stomach. However, little is known about their biological functions as well as the occurrence in other animal cells and tissues, due to limitations of analytical methods to detect minute amounts of sulfated Sia. Therefore, an objective of this study is to develop methods for detecting the sulfated Sia as well as probes for pursuing their functions. First, we examined if the fluorometric HPLC analysis was applicable as a sensitive chemical detection. Samples were hydrolyzed under acidic conditions and the liberated, sulfated Sia was derivatized with DMB (1,2-diamino-4,5-methylenedioxybenzene), followed by a reverse-phase HPLC analysis. It was demonstrated that 8-O- and 9-O-sulfated Neu5Ac, Neu5Gc and KDN could be separated on the HPLC. At least 100 fmol of the sulfated Sia could be detected by this method. Second, we generated monoclonal antibodies against sulfated Sia using glycoconjugates isolated from sea urchin gametic cells as immunogens. Three monoclonal antibodies were obtained: mAb.3G9, specific to 8-O-sulfated Neu5Ac; mAb.2C4, specific to 8-O-sulfated Neu5Ac or 8-O-sulfated Neu5Gc, and mAb.H4C2, specific to 8-O-sulfated (Neu5Ac)2 or 8-O-sulfated (Neu5Gc)2. Finally, using these methods and the antibodies, we showed that both glycoproteins and glycolipids from sea urchin sperm exclusively contained 8-O-sulfated Neu5Ac, while those from the eggs contained 8-O-sulfated Neu5Gc. Furthermore, we compared the content of 8-O-sulfated Neu5Gc in eggs of *Hemicentrotus pulcherrimus* with that of *Strongylocentrorus purpuratus*, and showed that 8-O-sulfated Neu5Gc was more enriched in *S. purpuratus* than in *H. pulcherrimus*. Immunofluorescent microscopic observations showed that the sulfated Neu5Gc was present not only in the egg surface but also in the egg cytoplasm.

(330) **In vivo Inactivation of Siglec-2 Ligand Formation in Mouse**

Yuko Naito[1], Hiromu Takematsu[1], Manabu Sugai[2], Shigeyoshi Itohara[3], Toshisuke Kaeasaki[4], Akemi Suzuki[5] and Yasunori Kozutsumi[1,5]

[1] Graduate School of Biostudies, Kyoto University, Kyoto, 606-8501, Japan, [2] Center for Molecular Biology and Genetics, Kyoto University, Kyoto, 606-8501, Japan, [3] Brain Science Institute, RIKEN, Wako 351-0198, Japan, [4] Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, 606-8501, Japan, [5] Supra-biomolecular System Group, RIKEN Frontier Research System, Wako 351-0198, Japan.

Sialic acid is a family of nine-carbon sugar that occupies surface of mammalian cells in their glycans of glycoproteins and glycolipids. Not only it participates to maintain the negative charge of cellular membrane or glycoconjugates, but also fine sugar moieties including sialic acid modification are shown to be involved in the molecular recognition events in wide variety of systems, i.e. immune system. Here we report the B cell phenotypes of the mice with disrupted CMP-N-acetylneuraminic acid (Neu5Ac) hydroxylase gene, which convert CMP-Neu5Ac to CMP-N-glycolylneuraminic acid (Neu5Gc) and is responsible for the biosynthesis of one of the major modification of sialic acid. The mouse showed total lack of the Neu5Gc in all tissues examined thus confirmed the total dependence on CMP-Neu5Ac hydroxylation for the Neu5Gc biosynthesis in cell. Lack of Neu5Gc prompted us for the further analyses of their B cell phenotypes since mouse B cell antigen CD22 (Siglec-2) requires NeuGc for its proper binding to its sialylated ligands. Indeed, CMP-Neu5Ac hydroxylase disrupted mouse lacked proper ligand formation on B cells. To address the question how lectin activity of the CD22 (Siglec-2) affects the function of CD22 as a B cell receptor signaling modulator, we assessed the consequence of Neu5Gc disruption in B cell biology. Even though the development of mature B cells appeared normal, analyses of the B cell functions in this CD22-ligand disrupted mouse showed hyper-responsive B cell phenotypes in T-independent immune response when immunized. This phenotype was explained by hyper proliferative response of Neu5Gc disrupted B cells in vitro culture system using anti-IgM or LPS as stimulation reagent. Collectively, we concluded that the recognition of the properly sialylated ligand by CD22 is required for its function as a B cell antigen receptor signaling negative modulator in vivo.

(331) **Occurrence and Characterization of a Novel Sulfated α2,9-Linked Polysialic Acid-Containing Glycoprotein in Sea Urchin Sperm Flagellum**

Shinji Miyata[1,2], Chihiro Sato[1,2] And Ken Kitajima[1,2,3]

[1] Biosci. Biotech. Center, Nagoya Univ., [2] Grad. Sch. Bioagr. Sci., Nagoya Univ., [3] Inst. Adv. Res., Nagoya Univ..

Previously, we showed an enrichment of a sulfated ganglioside HSO_3→8Neu5Acα2→8Neu5Acα2→6GlcCer in lipid rafts of sea urchin sperm. The sulfated ganglioside is bound by egg 350 kDa sperm binding protein (SBP), and this binding is considered to be important for the sperm-egg interaction at fertilization. We also demonstrated that a HSO_3→8Neu5Ac glycotope was expressed on several glycoproteins of sea urchin sperm. To gain a further insight into the biological significance of the HSO_3→8Neu5Ac glycotope, we began to study HSO_3→8Neu5Ac glycotope on glycoproteins. In this study, we report the occurrence of a novel HSO_3→8Neu5Ac glycotope-containing polysialic acid structure on a sea urchin sperm glycoprotein.

First, the major sialic acid-containing glycopeptide fraction (designated SGP) was purified from the exhaustive Actinase E digest of sea urchin sperm. The structure of glycan chains of SGP was determined by various chemical analyses as follows: HSO_3→8Neu5Acα2→9(Neu5Acα2→9)$_{n-2}$Neu5Acα2→6GalNAcα1→Ser/Thr (n=about 15). [Miyata *et al.* (2004) *Glycobiology* **14**, 827-840]. This is the first demonstration of the occurrence of polysialic acids in animal sperm. Notably, the presence of the α2,9-linked polysialic acid structure in glycoprotein has been recently suggested in mouse neuroblastoma cells. Thus, the α2,9-linked polysialic acid structure in glycoproteins may possibly occur more frequently than ever recognized, like the α2,8-linked polysialic acids that are commonly distributed from bacteria to animals.

Second, to identify the carrier protein of the unique polysialic acid, Western blot analysis using an antibody against the HSO_3→8Neu5Ac glycotope was performed. The 220, 137, 130, 80, 60, and 40-80 kDa glycoproteins were shown to contain the HSO_3→8Neu5Ac glycotope. On SDS-PAGE/fluorometric HPLC analysis, the α2,9-linked Neu5Ac residues were exclusively detected in the 40-80 kDa glycoprotein. Therefore, it is indicated that the 40-80 kDa glycoprotein is a sialic acid-rich glycoprotein (~ 60% by weight) and that most Neu5Ac residues are present as α2,9-linked Neu5Ac. We also demonstrated that this glycoprotein is localized in the flagellum of sperm. The 40-80 kDa glycoprotein was purified by DEAE-Toyopearl chromatography and WGA-affinity chromatography. The purified 40-80 kDa glycoprotein was hydrolyzed with 0.1 N trifluoroacetic acid at 80 ºC for 1 h to remove the α2,9-linked Neu5Ac residues. This treatment gave a single, major 24 kDa component on SDS-PAGE. The amino acid sequence analyses of the 24 kDa component-derived fragments were performed. The sequences for the fragments appeared not to match known sequences in the data bank, thus indicating that the 40-80 kDa glycoprotein is a novel glycoprotein. The molecular cloning of this glycoprotein is currently under way in our laboratory. This work has been supported in part by CREST of JST.

(332) **Heparan sulfate 6-*O*-sulfotransferase-1 (HS6ST-1) Deficient Mice: Defective Heparan Sulfate Biosynthesis Caused Runting and Perinatal Lethality**

Hiroko Habuchi[1], Naoko Nagai[1], Noriko Sugaya[1], Fukiko Atsumi[2] and Koji Kimata[1]

[1] Institute for Molecular Science of Medicine,
[2] Laboratory Animal Research Center, Aichi Medical University, Yazako, Nagakute, Aichi 480-1195, Japan.

Heparan sulfate proteoglycans (HSPGs) are present ubiquitously on the cell surface, extracellular matrix and basement membranes and play crucial roles in development, physiological phenomena and pathogenic processes. These biological functions are achieved through the interaction with HS chains and the huge numbers of ligands such as heparin-binding growth factors (HBGFs), and basement membrane components. In some cases, HS interacts with the ligands through specific monosaccharide sequences characterized by the sulfation pattern and isomer of hexuronic acid residue. The divergent structures of heparan sulfate are synthesized by the successive actions of heparan-sulfate modifying enzymes (*N*- and *O*-sulfotransferases and C5-epimerase). The 6-*O*-sulfation is carried out by three isoforms of heparan sulfate 6-*O*-sulfotransferase (HS6ST-1, -2, -3) and one spliced form of HS6ST-2 (HS6ST-2S). To elucidate the *in vivo* roles distinctive of each HS6ST isoforms, we firstly generated mice with a targeted deletion of the HS6ST-1 gene. Most of HS6ST-1 null mice died from E15.5 day to perinatal stage. A few percent of the homozygotes were viable and fertile, but the growth rate was severely retarded. Remarkable reduction of GlcNAc-6S residue and HexA-GlcNS(6S) residue of HS was observed in the various organs such as liver, kidney and lung, which were shown to express HS6ST-1 strongly, whereas reduction of IdoA(2S)-GlcNS(6S) residues was only slightly. On the other hand,HexA(2S)-GlcNS residues rather increased. Histological analysis revealed that the morphology of fetal microvessels in labyrinth zone of placenta was abnormal, suggesting that alteration of the HS resulted in runting and perinatal lethality. The retina disorganization and lung defects were also observed. To clarify the possibility that those defects are due to the abnormal regulation of the signaling of HBGFs, the responses of fibroblasts obtained from HS6ST-1$^{-/-}$ embryo to various HBGFs are under investigation.

(333) **NCAM Polysialylation by ST8SiaII and ST8SiaIV**

Martina M¸hlenhoff[1], Imke Oltmann[1], Sebastian Galuska[2], Birgit Weinhold[1], Rudolf Geyer[2], Hildegard Geyer [2] and Rita Gerardy-Schahn[1]

[1] Abteilung Zellul‰re Chemie, Medizinische Hochschule Hannover, Hannover, Germany,
[2] Institut f¸r Biochemie, Medizinische Fakult‰t, Universit‰t Giessen, Giessen, Germany.

Polysialylation of the neural cell adhesion molecule, NCAM, is mediated by the activity of two separate gene products the polysialyltransferases ST8SiaII and ST8SiaIV. The functional mechanisms of both enzymes as well as their time and tissue specific expression patterns are matter of intensive biochemical studies. In the majority of tissues ST8SiaII and IV are co expressed, however, the expression levels are differentially regulated in the course of embryonic development and recent studies carried out with knock-out mice expressing only one of the two polysialyltransferases have clearly demonstrated that ST8SiaII is predominant in the young (Angata et al., 2004, J. Biol. Chem. 279, 32603) while ST8SiaIV is the most important enzyme in the adult animal (Eckhardt et al., 2000, J. Neurosci. 20, 5234). This study has profited from the availability of mice expressing only one (ST8SiaII knock-out; ST8SiaIV knock-out) or none (ST8SiaII/ST8SiaIV double knock-out) of the two polysialyltransferases. Brain samples have been collected from all available genotypes at different time points between postnatal day 1 up to adulthood. In a comparative study polysialic acid and NCAM expression was analysed at both quantitative and qualitative level using a combination of immunological and biochemical techniques. The in vivo analysis of the polysialylation competence of the two polysialyltransferases highlights the essential role of ST8SiaII during postnatal brain development. Clear differences in the polysialylation pattern were observed in mice lacking either ST8SiaII or ST8SiaIV, revealing a new regulatory level in NCAM-polysialylation.

(334) **Expression of Gal 3-O-sulfotransferase GP3ST Suppresses Adhesive and Metastatic Properties of Human Lung Cancer Cells**

Naoki Ikeda[1], Naoyuki Taniguchi[2] and Koichi Honke[1]

[1] Department of Molecular Genetics, Kochi University Medical School, Nankoku 783-8505, Japan,
[2] Department of Biochemistry, Osaka University Medical School, Suita 565-0871, Japan.

The negative charges of sulfate group and sialic acid are thought to serve as an adherent force in interactions with a variety of functional molecules, which include growth factors, cellular adhesion molecules, and extracellular matrix proteins. Previous studies have demonstrated that the expression of the sulfo-3Galβ1-3(Fucα1-4)GlcNAc-R (3'-sulfo-Le(a)) decreases with increasing depth of invasion of human colon carcinomas, and human colon carcinoma cells expressing the 3'-sulfo-Le(a) epitope show a lower tumorigenicity in nude mice. However, the physiological roles of 3'-sulfated Lewis epitopes remain to be elucidated. We have reported on the cDNA cloning of a Gal 3-O-sulfotransferase GP3ST that acts on both type 1 (Galβ1-3GlcNAc-R) and type 2 (Galβ1-4GlcNAc-R) chains, based on its similarity to glycolipid 3-O-sulfotransferase. When the GP3ST gene was introduced into Chinese hamster ovary cells expressing α1,3-fucosyltransferase, 3'-sulfo-Le(x) antigen appeared and expression of sialyl-Le(x) antigen was attenuated, indicating that GP3ST and α2,3-sialyltransferase compete for the common precursor. The aim of this study was to determine whether expression of GP3ST can suppress adhesive and metastatic properties of human lung cancer cells. The human lung cancer cell line ABC-1 expresses sialyl-Le(x), but does not express 3'-sulfo-Le(x). Overexpression of the GP3ST gene in ABC-1 cells resulted in a substantial reduction of sialyl-Le(x) that correlated with an increase of expression of 3'-sulfo-Le(x) antigen. The reduction of sialyl-Le(x) antigen expression was associated with a suppression of adhesive property to E-selectin and lung metastasis of ABC-1 cells after tail injection into nude mice. This study provides evidence that the expression level of Gal 3-O-sulfotransferase may regulate the expression of sialy-Le(x) antigen and consequently could play an important role in metastatic properties of human lung cancer cells.

(335) **Up-regulated Expression of α5β1 Integrin and Reduced Cell Motility in Gnt-V Deficient Mouse Embryo Fibroblasts**

Hua-Bei Guo, Intaek Lee, Beau T. Bryan, Gerardo Alvarez-Manilla and Michael Pierce

Complex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology,
University of Georgia, Athens, Georgia 30602.

Tumor progression is associated with aberrant glycan expression on the cell surface and altered cell adhesive properties. A common glycan whose expression often increased in carcinomas is the β1,6-linked GlcNAc on N-glycans, synthesized by GnT-V. Altered expression of this branched glycan on cell adhesion receptors has been associated with altered adhesion, motility, and intracellular signaling. The progression of pmt oncogene-induced mammary carcinomas in GnT-V (-/-) mice was significantly retarded compared to those in GnT-V (+/-) mice [Granovsky et al., Nature Med. 6, 306, 2000]. The mechanism of how deletion of GnT-V down-regulated tumor progression was explored using mouse embryonic fibroblasts (MEFs) isolated from GnT-V null and heterozygote mice. Compared to heterozygote MEFs, null MEFs displayed significantly decreased cell growth, decreased cell survival after 6 days in serum-free media, and enhanced cell adhesion to fibronectin-coated plates. Using wound healing and a transwell cell migration assay toward fibronectin, migration was markedly inhibited in GnT-V-deficient cells. Null MEFs also showed increased FAK tyrosine phosphorylation, increased focal adhesions/cell, determined by staining with antibodies to talin, vinculin and paxillin, and temporally retarded fibronectin-induced cortical F-actin rearrangement. These characteristics are all consistent with the observed decreased cell motility on fibronectin. Expression of GnT-V cDNA in GnT-V-null cells reversed these abnormal characteristics, indicating the direct involvement of N-glycosylation events in these phenotype changes. Surprisingly, null MEFs displayed significantly increased mRNA, protein, and cell surface expression levels of both α5 and β1 integrin subunits compared to wild-type MEFs. α5β1 expression levels in the null MEFs returned to wild-type levels after expression of GnT-V cDNA, confirming that the increased α5β1 expression in null MEFs was due to changes in GnT-V expression levels. These results demonstrate that deletion of MEFs GnT-V results in up-regulated levels of both total cellular and cell surface α5β1 fibronectin receptors, consequently increasing fibronectin-mediated cell-matrix adhesion and inhibiting migration.

(336) **Expression and Interaction of Sulfoglucuronyl (HNK-1) Carbohydrate, Amphoterin and Receptor for Advanced Glycation End Products (RAGE) in Mouse Cerebellum**

Denise K. H. Chou and Firoze B. Jungalwala

Department of Neurobiology, E. K. Shriver Center at University of Massachusetts Medical School, Waltham MA USA.

Sulfoglucuronyl (HNK-1) carbohydrate (SGC) has been shown to interact with Amphoterin, a neurite outgrowth promoting protein. In vitro studies with neuroblastoma cell cultures suggested that Amphoterin initiated neurite out growth by its interaction with a membrane protein, receptor for advanced glycation end products (RAGE) by signal transduction via GTPases, Rac and Cdc42. Rac and Cdc42 recognize the actin cytoskeleton and promote actin polymerization required for neurite extension. The developmental expression of SGC, Amphoterin and RAGE, was studied in prenatal and postnatal mouse cerebellum to establish their cellular and sub-cellular localization and function in vivo. The amount of RAGE in the cerebellum increased with age. RAGE was expressed prenatally in the external germinal layer and postnatally in the plasma membranes of the granule neurons of the external and internal granule cell layers and in Purkinje cells. Immunocytochemical analysis by high magnification confocal microscopy showed that RAGE was co-expressed with Amphoterin and SGC in the cell surfaces of granule neurons. This co-localization of RAGE, Amphoterin and SGC was confirmed in isolated and cultured granule neurons and in migrating granule neurons in explant cultures. Anti-RAGE antibodies inhibited neurite out growth and cell migration in explant and slice cultures, similar to anti-Amphoterin and anti-SGC antibodies shown previously (Chou et al., J Neurosci Res. 59:188-201, 2000). Previously we have shown that after retinoic acid treatment, embryonal carcinoma P19 cells differentiated into neuron-like cells and concomitantly induced expression of SGC, Amphoterin, and RAGE on their cell membranes leading to extensive neurite outgrowth (Chou et al., J Neurochem. 86:917-931, 2003). Direct interactions between SGC-proteins and Amphoterin and between Amphoterin and RAGE was shown by co-immuno-precipitation of the proteins with appropriate antibodies, from retinoic acid-treated P19 cells and from isolated granule neurons of postnatal day 8 cerebellum. The results suggest that RAGE could act as a signaling molecule for neurite outgrowth and cell migration by its interaction with Amphoterin and that of Amphoterin with SGC in vivo.

(337) **Function of the Drosophila Sulfotransferase Pipe in the salivary Gland and the Ovary**

Dave Stein[1], Xianjun Zhu[1], Jason S Goltz[1], Jonaki Sen[2], Zhenyu Zhang[1] and Leslie M Stevens[1]

[1] Section of Molecular Cell and Developmental Biology, University of Texas at Austin,

[2] Department of Molecular Genetics, Albert Einstein College of Medicine.

The Drosophila pipe gene encodes up to ten related proteins, generated by alternative RNA splicing, all with amino acid sequence similarity to vertebrate glycosaminoglycan sulfotransferases. The localized expression of one of the Pipe protein isoforms in ventral follicle cells of the Drosophila ovary is a key event in the correct establishment of embryonic dorsal-ventral polarity, suggesting that Pipe-mediated sulfation of a key glycan in the nascent secretory pathway of ventral follicle cells is an essential step in embryonic pattern formation. While the function of the other nine Pipe isoforms has remained mysterious, the expression of several of the Pipe isoforms in the embryonic salivary gland has suggested a role in the formation or function of that organ. A unique semi-lethal allele of pipe leads to disruption of normal salivary gland morphology and function. A similar phenotype is observed in mutants affecting windbeutel, another dorsal group gene that has previously been demonstrated to be required for the correct subcellular localization and function of Pipe. While characterizing the effects of pipe and windbeutel mutations on salivary glands we have detected a novel macromolecule in the lumen of embryonic salivary glands that stains with alcian blue, a histochemical stain that has been used extensively to detect sulfated glycoproteins such as proteoglycans and mucins. Lumenal alcian blue staining of embryonic salivary glands requires the function of genes involved in the metabolism of the universal sulfate donor PAPS, suggesting that the staining material may be a sulfated product of Pipe enzymatic action. Surprisingly, alcian blue staining of salivary glands does not require the function of genes previously implicated in the formation of glycosaminoglycans in Drosophila, suggesting that the stained material does not represent a conventional glycosaminoglycan. We will also report on our studies of the role of Pipe and glycan modification in the establishment of embryonic dorsal-ventral polarity.

(338) **Monocyte Differentiation Triggers Upregulating of Lysosomal Sialidase Neu1 and its Targeting to the Plasma Membrane.**

Alexey V. Pshezhetsky[1], Feng Liang[1], Volkan Seyrantepe[1], Karine Landry[1], Rasheed Ahmad[1], Ali Ahmad[1] and Nicholas M. Stamatos[2]

[1] Hopital Sainte-Justine and Departement de pediatrie, Universite de Montreal, Montreal, Quebec H3T 1C5 Canada , [2] Institute of Human Virology, University of Maryland, Baltimore, Maryland 21201, Division of Infectious Diseases, Department of Medicine, University of Maryland Medical Center, Baltimore, Maryland.

Sialidase (neuraminidase) Neu1, encoded by the NEU1 gene in the MHC locus on human chromosome 6 catalyzes lysosomal catabolism of sialylated glycoconjugates. Neu1 mRNA, protein and activity are increased 12-15 fold when freshly-isolated, human monocytes differentiate in culture into macrophages. Neu1 is also increased ~3-fold during the PMA-induced differentiation of monocytic cell line THP-1. In both cell models majority of the newly-synthesized Neu1 instead of being targeted to lysosomes is found on the cell surface. We investigated the mechanisms controlling the up-regulation and targeting of Neu1 and found a 550-bp fragment of the NEU1 gene promoter containing several potential AP-1 binding sites, which transcription activity in THP-1 cells was significantly induced by PMA. The promoter activity was also potently induced by the pro-inflammatory factor, TNF-a, that activates AP-1 expression and reduced by AP-1 inhibitors: curcumin and N-acetylcysteine. We further demonstrated that redistribution of Neu1 from the lysosomes to the cell surface is accompanied by similar sorting of the lysosomal carboxypeptidase, cathepsin A. Since in the lysosome this cathepsin forms a complex with Neu1 activating and protecting it we speculate that both proteins are first targeted to the lysosome and then as a complex to the plasma membrane. In accordance with this hypothesis we found that fluorescent dextran stored in lysosomes of THP-1 cells was sortied to the cell surface during PMA-induced differentiation. We also found that at all stages of differentiation Neu1 was co-localized with MHC II and with internalized antigens suggesting the existence in macrophages of a sorting pathway out of the lysosomes potentially used for antigen presentation.

(339) **Terminal Glycans in Evolution and Speciation**

Suvarna Deshmukh, Y.Lucie Kim, Mallika Dhawan, Anil Challa, Ajit Varki and Nissi Varki

Glycobiology Research and Training Center, 9500 Gilman Drive, UC San Diego, La Jolla, CA 92093-0687.

Organs from different vertebrate species appear generally similar on simple histologic examination. However, there are established inter-species differences in the histological architecture of organs from different vertebrates. For example, there are differences in the organization of lymphoid follicles in mouse, rat and human spleen. While there is a lack of obvious mucin-containing goblet cells in the bronchial epithelium of unstimulated mice, such cells have been described in guinea pigs, a closely related species. Encircling collagen bundles delineates the lobules in porcine liver, a feature not evident in livers of many other species. In a like manner, preliminary evidence suggests that there may be significant inter-species differences in glycosylation. We are using a series of natural and recombinant glycan-recognizing probes (GRPs) to characterize the cell-type specific expression of multiple kinds of terminal glycan structures within the major tissues of multiple vertebrate species. Plant lectins have been particularly powerful tools to explore glycan structures. Because of the specificity of each lectin towards particular structures, even isomeric glycans with identical sugar compositions can be distinguished. Likewise the host-recognizing proteins of some microbes (e.g., hemagglutinins of viruses) can have exquisite specificity for recognition of terminal glycans, and recombinant versions of such molecules can be used as GRPs. Using a limited set of such GRPs, we have generated a baseline description of the patterns of expression of various glycan termini in the organs and tissues of the normal laboratory mouse, and in the process uncovered clues to other potential biological consequences of the deficiency of two particular sialyltransferase genes. Taking advantage of the availability of different inbred strains of mice, we have also found that is relatively little intra-species variation in terminal glycosylation. In contrast, comparisons with the rat showed remarkable differences. Likewise, when comparing the terminal glycans in organs from humans and the great apes, we observed a difference in the expression of a particular linkage of sialic acids in multiple tissues, which represents an example of a human -specific change in sialic acid biology. We are now analyzing sections from organs of different multiple vertebrate species (mice, rats, hamsters, zebrafish) and are observing major variations in expression of binding of different GRPs to similar organs in the different species. These data suggest that terminal glycosylation patterns are diverging rapidly during evolution, but that the

pattern for each species might be fixed at the time of speciation. On the other hand, certain types of terminal glycosylation appear to be highly conserved over long evolutionary times, e.g., the expression of sialic acid O-acetylation and the absence of N-glycolylneuraminic acid in neural cells. As the dataset increases, we hope to define more such examples where conservation in the face of such a rapidly diverging glycomic changes might help to predict roles of glycans in critical endogenous functions.

(340) **β3GnT1 Function in Sensory Neuron Pathfinding and Survival**

Timothy R. Henion[1,2], Denitza Raitcheva[1], Franziska Biellmann[3], Thierry Hennet[3] and Gerald A. Schwarting[1,2]

[1] Shriver Center for Mental Retardation, Waltham, MA, USA, [2] Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA, USA, [3] Institute of Physiology, University of Zurich, Zurich, Switzerland.

A specific role for carbohydrates in establishing sensory axon connections with target tissues has emerged from the analysis of β3GnT1$^{-/-}$ mice. Olfactory sensory neurons (OSNs) expressing the same odorant receptor are dispersed in the nasal cavity but extend axons that collectively converge to a single synaptic locus in each olfactory bulb (OB), termed a glomerulus. Terminal N-acetyllactosamine (LN) glycans recognized by the mAb 1B2 are expressed by OSNs during axon extension to the OB. Lycopersicon esculentum agglutinin (tomato lectin) also binds OSNs and their glomerular projections with high specificity, which implies that 1B2 may be recognizing poly-N-acetyllactosamine glycans. Mice genetically deficient for the glycosyltransferase β3GnT1 lose LN expression on OSNs and have severely disorganized OB projections. At postnatal day 1, most axons expressing the mature olfactory neuron marker OMP fail to establish glomerular contact with OB mitral cells. This defect is paralleled by a decrease in both OE thickness and number of OMP^{+} cells, suggesting that an absence of trophic support from failed synaptic contact may underlie the increased OSN apoptosis observed in null mice. Subsets of OSNs expressing distinct odorant receptors are differentially affected by LN loss, potentially reflecting differences in LN expression. The defects in OB organization correlate with poor performance of β3GnT1$^{-/-}$ mice in olfactory food-finding tasks. In addition to these olfactory defects, pheromone-receptive vomeronasal neurons and nociceptive dorsal root ganglion cells also express 1B2-reactive LN which is β3GnT1-dependent, suggesting that LN glycans may be involved in axon pathfinding in several sensory systems.

(341) **Optimized Sialic Acid Method for Monitoring Monoclonal Antibody Sialic Acids**

Andrea Beard, Paul Kodama and Wei-Chun (Wesley) Wang

Amgen Inc., 1201 Amgen Court West, Seattle, WA98119-3105 USA.

A highly sensitive, fluorescent labeled off the shelf chemical sialic acid method has been developed and optimized to monitor the sialic acid content of monoclonal antibodies. Monoclonal antibody N-linked glycans are complex type bi-antennary structures with 0 to 2 sialic acid residues and possible O-linked glycans with 1 to 2 sialic acids. This small amount of sialic acid quatitation presented a special challenge for quality control laboratory operations. The optimized method's quick and straightforward procedure has the sensitivity and specificity required to assess the N-acetylneuraminic acid (NANA) and N-glycolylneuraminic acid (NGNA) content of a monoclonal antibody with a high level of accuracy and precision. This poster summarizes the optimization of this method.

(342) **A homolog of N-acetylglucosaminyltransferase V (GnT-V) Shows High Expression Levels in Mouse and Human Brain**

Jin-Kyu Lee[1], Karen Abbott[1], Karolyn Troupe[1], Mika Kaneko[1], Rick Matthews[2] and Michael Pierce[1]

[1] Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602,

[2] Department of Neurobiology, Yale University School of Medicine, New Haven, CT 06520.

A homolog of GnT-V (UDP-N-acetylglucosamine:alpha(1,6)-D-mannoside beta(1,6)-N-acetylglucosaminyltransferase) exists in both humans and mice and has been termed both GnT-VB and GnT-IX. The human GnT-VB shows 41% amino acid identity and 53% similarity with human GnT-V, while mouse GnT-VB has 42% identity and 70% similarity with mouse GnT-V. Despite these high degrees of homology between GnT-V and GnT-VB, both mouse and human GnT-VB show a requirement for divalent cation for full activity, by contrast to GnT-V from both species. Significant differences are also apparent in the affinity of the GnT-VB enzymes toward many acceptor substrates compared to the GnT-V enzymes. Transcripts of human and mouse GnT-VB that contain a 6 bp insertion between exons 10 and 11 are also found in cells and in EST databases. When expressed in HEK cells and assayed in the presence of Mn++, human and mouse +6 bp forms both show very low levels of activity compared to the -6 bp forms. Real-time PCR results demonstrate that GnT-V is basally expressed in most human and mouse tissues; by contrast, human and mouse GnT-VB is expressed mainly in brain and testis. We have examined the expression of GnT-V and GnT-VB during development and have used a variety of agonists to challenge cells that express these enzymes to better understand the different mechanisms that regulate their expression.

(343) **Distinct and Collaborative Roles of Drosophila Ext Family Tumor Suppressors in Heparan Sulfate Proteoglycan Biosynthesis and Growth Factor Signaling**

Chun Han[1,2], Tatyana Y. Belenkaya[2] and Xinhua Lin[1,2]

[1] Molecular and Developmental Biology Program, College of Medicine, University of Cincinnati,

[2] Division of Developmental Biology, Cincinnati Children's Hospital Medical Center.

Proteoglycans play many critical roles in animal development. Especially, heparan sulfate proteoglycans (HSPG) have been implicated in regulating several essential developmental signal transduction pathways including Wnt/Wingless (Wg), Hedgehog (Hh) and TGF-¶¬/Decapentaplegic (Dpp) signaling. HSPG consists of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are attached. The formation of HS GAG chains is catalyzed by glycosyltransferases encoded by members of the EXT family of putative tumor suppressors linked to hereditary multiple exostoses (HME). Previous studies in Drosophila demonstrated that tout-velu (ttv), the Drosophila EXT1 is required for Hh movement. However, the functions of other EXT family members are unknown and the core proteins of HSPGs responsible for growth factor signaling have not been identified. Here we have identified and isolated mutations in other two members of Drosophila EXT family genes, which are named as sister of tout-velu (sotv) and brother of tout-velu (botv) encoding Drosophila homologues of vertebrate EXT2 and EXT-like 3 (EXTL3), respectively. Both Hh and Dpp signalling activities as well as their ligand distributions are defective in cells mutant for ttv, sotv or botv in the wing disc. Surprisingly, while Wg distribution is abnormal in ttv, sotv and botv, Wg signalling is only defective in botv or ttv-sotv double mutant, but neither in ttv nor in sotv alone, suggesting that Ttv and Sotv are redundant in Wg signaling. We show further that Ttv and Sotv form a complex and are co-localized in vivo. These results provide evidence that all three Drosophila EXT proteins play distinct and collaborative roles in the biosynthesis of HSPGs and growth factor signaling. Finally, we demonstrate that glypicans Dally and Dally-like (Dly) are the substrates of EXT proteins and mediate the function of EXT genes in growth factor signaling.

(344) **Targeted Disruption of the Walker-Warburg Syndrome Gene *Pomt1* in Mouse Results in Embryonic Lethality**

Tobias Willer[1], BelÈn Prados[2], Juan M FalcÛn[2], Ingrid Renner-Mueller[3], Gerhard KH Przemeck[4], Mark Lommel[1], Antonio Coloma[2], M Carmen Valero[2], Martin HrabÈ de Angelis[4], Widmar Tanner[1], Eckhard Wolf[3], Jes˙s Cruces[2] and Sabine Strahl[1]

[1] Lehrstuhl f¸r Zellbiologie und Pflanzenphysiologie, Universit‰t Regensburg, D-93040 Regensburg, Germany,

[2] Departamento de Bioqu'mica, Instituto de Investigaciones BiomÈdicas "Alberto Sols" CSIC-UAM, Facultad de Medicina, Universidad AutÛnoma de Madrid, 28029, Madrid, Spain, [3] Department of Molecular Animal Breeding and Biotechnology, Gene Center of the Ludwig-Maximilian University, D-81377 Munich, Germany, [4] GSF-National Research Center for Environment and Health, Institute of Experimental Genetics, D-85764 Neuherberg, Germany.

O-mannosylation is an important protein modification in eukaryotes that is initiated by an evolutionarily conserved family of protein O-mannosyltransferases. The first mammalian protein O-mannosyltransferase gene described was the human *POMT1*. Mutations in *hPOMT1* gene are responsible for Walker-Warburg syndrome (WWS), a severe recessive congenital muscular dystrophy associated with defects in neuronal migration that produce complex brain and eye abnormalities. During embryogenesis the murine *Pomt1* gene is prominently expressed in the neural tube, in the developing eye and mesenchyme. These sites of expression correlate with those in which the main tissue alterations are observed in WWS patients. We have inactivated a *Pomt1* allele by gene

targeting in embryonic stem cells and produced chimeras transmitting the defect allele to offspring. While heterozygous mice were viable and fertile, the total absence of *Pomt1*$^{-/-}$ pups in the progeny of heterozygous intercrosses indicated, that this genotype is embryonic lethal. An analysis of the mutant phenotype revealed that homozygous *Pomt1*$^{-/-}$ mice suffer developmental arrest around day 7.5 of embryogenesis (E7.5), and die between E7.5 and E9.5. The *Pomt1*$^{-/-}$ embryos present defects in the formation of Reichert's membrane, the first basement membrane to form in the embryo. The failure of this membrane to form appears to be the result of abnormal maturation of dystroglycan that may impair recruitment of laminin, a structural component required for the formation of Reichert's membrane in rodents. The targeted disruption of *mPomt1* represents the first example of an engineered deletion of a known glycosyltransferase involved in O-mannosyl glycan synthesis.

(345) **Changes in the Distribution of Major Brain Gangliosides During Vertebrate Evolution**

Marija Heffer-Lauc[1], Ana Mojsovic[2], Domagoj Dikic[3], Ivan Birus[4], Ronald L. Schnaar[5] and Gordan Lauc[4]

[1] University of Osijek School of Medicine, Department of Biology, J. Huttlera 4, 31000 Osijek, Croatia, [2] School for Health Studies, Mlinarska 38, 10000 Zagreb, [3] University of Zagreb, Faculty of Science, Department of Animal Physiology, Roosveltov trg 6, 10000 Zagreb, Croatia, [4] University of Osijek School of Medicine, Department of Chemistry and Biochemistry, J. Huttlera 4, 31000 Osijek, Croatia, [5] Departments of Pharmacology and Neurosciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

Gangliosides are highly conserved glycoconjugates present in all vertebrates. Despite the fact that they are ubiquitously present in brains of all animals, very little is known about their functions. Aiming to aid the understanding of the evolution of functional roles of gangliosides, we have studied the distribution of the major brain gangliosides (GM1, GD1a, GD1b, GT1b) in brains of trout, carp, frog, snake, chicken, bat, rat, ferret, rabbit and cat. Highly specific monoclonal antibodies were used to detect ganglioside patterns in paraformaldehyde-fixed brains. GT1b was found in brains of all studied animals where it was present on cell bodies of virtually all neurons. GD1a was apparently not present in the fish brain, while in frog and snake brains it was present only on neurons in frontal telencephalon. In other studied animals it was present on neuronal bodies in all brain regions. GD1b and GM1 were not detected in fish, frog and snake brains. In other studied animals they were present in a species-specific distribution patterns. In most animals, the fiber system of the telencephalon contained some GD1a, GD1b and GT1b, while the fiber system of the cerebellum was mostly negative for these gangliosides, particularly GD1a. Interestingly, in all brains where GM1 was present, it was restricted strictly to fiber systems. Although the expression pattern of gangliosides appears to be quite diverse, once developed, these patterns seem to be conserved in further evolutional development, indicating that specific gangliosides might have the same functional roles in various animals.

(346) **Role of Chondroitin Sulfate Chains of Versican/PG-M In Regulation of Chondrogenesis as an Essential Factor for Mesenchymal Condensation**

Nobuhiro Kamiya[1,2], Hideto Watanabe[1], Hidekazu Takagi[1], Tamayuki Shinomura[3], Katsuji Shimizu[2] and Koji Kimata[1]

[1] Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi, Japan, [2] Department of Orthopaedic Surgery, Gifu University, Gifu, Japan. , [3] Department of Hard Tissue Engineering, Tokyo Medical and Dental University, Tokyo, Japan..

Cartilage formation in limb buds, one of the most typical examples of organogenesis, starts with the condensation of chondrogenic mesenchymal cell, and this step is thought to be essential for subsequent skeletal development in vertebrates. Versican/PG-M is one of the major molecules expressed in the extracellular matrix (ECM) during the condensation, and a large chondroitin proteoglycan consisting of two globular domains at the N-terminal and C-terminal (G1 and G3 domain, respectively) and two chondroitin sulfate-attachment domains (CS-alpha and CS-beta), of which the latter two domains give unique properties to this proteoglycan in that multiple alternative splicing yields four variant forms with different numbers of attached chondroitin sulfate chains; V0 having CS-alpha and CS-beta, V1 having CS-beta, V2 having CS-alpha, and V3 having neither of the two. However, its role, especially as an environment for cells being condensed and the relationship between the unique spliced forms and their function have not been elucidated. Here we show several lines of evidence for essential roles of versican/PG-M in chondrogenic condensation using a new chondrocytic cell line, N1511. Chondrogenic stimuli (treatment with PTH/dexamethason/10% serum) induced a marked increase in the transcription and protein synthesis of versican/PG-M and subsequently in those of aggrecan and type II collagen, major chondrocytic cell products. Stable antisense clones for versican/PG-M, depending on suppression of the expression and deposition of versican/PG-M, showed different capacities for chondrogenesis, as indicated by the expression and deposition of aggrecan. The cells in the early stages of the culture only expressed V0 and V1 forms having more chondroitin sulfate chains among the four variants of versican/PG-M, and treatment of those cells with chondroitinase ABC suppressed subsequent chondrogenesis. Furthermore, forced expression of the variant V3 having no chondroitin sulfate chain disrupted the deposition and organization of native versican/PG-M (V0/V1) and other ECM molecules known to be expressed during the mesenchymal condensation, and resulted in the inhibition of subsequent chondrogenesis. These results suggest that versican/PG-M regulates the formation of the mesenchymal matrix and the onset of chondrocyte differentiation through the attached chondroitin sulfate chains.

(347) **The Sialic Acid Biology of Human Stem Cells: Basic Steps towards the Characterization and Manipulation of Glycosylation to Control Their Differentiation and Proliferation**

Adrienne Li[1], Zhiyun Wang[1], Michael P Murrell[2], Brent Cameron[1], Gautam Baskaran[1], Nicholas M Stamatos[3], Hai-Quan Mao[1] and Kevin J Yarema[1]

[1] Whitaker Biomedical Engineering Institute, The Johns Hopkins University, 3400 North Charles Street, Baltimore MD 21218, [2] Department of Biological Engineering, Massachusetts Instititute of Technology, 77 Massachusetts Avenue, Cambridge MA 02139 , [3] Institute of Human Virology, University of Maryland, 725 West Lombard Street, Baltimore MD 21201.

Stem cells have enormous potential to treat existing disease as well for the de novo construction of tissues and organs in tissue engineering applications. Both avenues of investigation would benefit from an increased understanding of the basic biology controlling the differentiation and proliferation of stem cells. Up to now, exploitation of glycosylation in stem cell research has been largely confined to its use as a diagnostic indicator of cellular status; efforts to actively intervene in the intracellular glycosylation machinery in order to direct desired cellular outcomes have lagged. Because of the incredible complexity of the glycosylation pathways of a cell, we initially focused our efforts on the subset of components responsible for sialic acid production. Our first task, described in this presentation, was to thoroughly characterize the expression of sialic acid processing enzymes by real-time PCR and compare these elements in stem cell and primary blood cell lines undergoing differentiation. In complementary experiments we analyzed these genes in cancer cell lines to gain a sense for their expression under conditions of controlled growth (stem cells) vs. uncontrolled growth (oncogenically-transformed cells). These efforts were intended to identify specific enzymes or the production of certain surface epitopes correlated with a desired (or undesired) outcome. In continuing work, the over-expression of recombinant forms of genes linked to beneficial outcomes or inhibition of genes linked to undesired outcomes by siRNA methods will establish whether these components play governing roles in cellular events or are merely secondary markers of biological processes under the control of other regulatory networks. A specific example where the former possibility is likely is the production of the GM3 and GD3 gangliosides from ceramide; these glycolipids are known to serve as a switch between senescence, proliferation, and apoptosis. Consequently, genetic or metabolic substrate based 'sialic acid engineering' methods under development in our laboratory aimed at controlling cellular production of GM3 and GD3 hold value as an important new tool for the tissue engineer.

(348) **Functional Analysis of *Drosophila* Sialyltransferase**

Kate Koles[1], Elena Repnikova[1], Efthimios M. C. Skoulakis[2], Carly Deguffroy[1], Jared Pitts[1] and Vlad Panin[1]

[1] Department of Biochemistry & Biophysics, Texas A&M University, College Station, TX 77843,

[2] Alexander Fleming Biomedical Research Centre, Vari, Greece 16602.

In deuterostomes, including mammalian organisms, sialic acids have been implicated in many important biological processes, such as the regulation of turnover of circulating glycoproteins and erythrocytes, pathogen-host recognition, immune system functioning, and nervous system development (1). At the same time, the evidence for sialylation in protostomes has been

scarce and somewhat controversial, while the functional role of sialic acids remains illusive. Recently, we have characterized a *Drosophila* sialyltransferase gene, *DSiaT* (2). We have demonstrated that this gene encodes a functional α2-6 sialyltransferase that is evolutionary related to the vertebrate ST6Gal sialyltransferase family. We have found that the expression of *DSiaT* is developmentally regulated and is restricted to certain cells within the central nervous system of Drosophila.
Our current experiments are aimed at the characterization of DSiaT function using molecular genetic approaches. We have generated mutations in *DSiaT* locus using the gene-targeting technique. The experiments revealed that *DSiaT* mutants have a behavioral phenotype, which indicates the involvement of this gene in neuronal functioning. We will present new data on DSiaT functioning in *Drosophila* development. This work was supported by NIH grant 1RO1GM069952-01.
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(349) **The role of O-fucosylation at EGF12 of Notch1 in Binding of the Notch Ligand Delta1**

Kazuhide Uemura, Shaolin Shi, Henry Kurniawan and Pamela Stanley
Department of Cell Biology, Albert Einstein College Medicine, New York, NY 10461.

Notch signaling determines cell fate, controls cell growth, and plays an important role in development. Notch receptors are cell surface glycoproteins containing *O*-fucose glycans on their EGF repeats. Mouse embryos lacking protein *O*-fucosyltransferase 1 (O-FucT-1) which transfers fucose to EGF repeats of Notch die at mid-gestation (1). Fringe proteins, well known modifiers of Notch signaling, are *O*-fucose specific β1,3-*N*-acetylglucosaminyltransferases. Moreover, the addition of Gal by β1,4Galactosyltransferase-1 to GlcNAc added by Fringe is required for Jagged1-induced Notch signaling in a CHO cell co-culture assay. We are investigating the mechanisms by which *O*-fucose glycans modulate Notch signaling. Using soluble Notch ligands Delta1-Fc and Jagged1-Fc and Pofut1$^{-/-}$ ES cells, we have shown that *O*-fucosylation by *O*-FucT-1 is required for ligand binding to Notch receptors. *O*-fucosylation occurs at multiple EGF repeats of Notch extracellular domain, including EGF12 which is part of the putative ligand binding domain (EGF11,12). Notch1 EGF11 has no *O*-fucose consensus site but Notch1 EGF12 acquires *O*-fucose at Thr 466 (2). In order to investigate the role of *O*-fucosylation at EGF12 in ligand binding to Notch1, we have used a mouse Notch1 EGF1-18 fragment (N1-18) with and without a mutation that precludes *O*-fucosylation in EGF12 (T12A mutation) (2). These fragments were produced from Lec1 CHO cells transfected with control vector or Manic Fringe, and affinity purified from conditioned medium using Ni-Sepharose. We have tested the inhibition of soluble Delta1-Fc binding to mouse Pofut1$^{+/+}$ ES cells or Lec1 CHO cells by wild type and mutant Notch fragments using FACS analysis. The wild type Notch1 fragment inhibited Delta1-Fc binding to ES cells and to Lec1 CHO cells in a dose dependent manner. Wild type Notch1 fragment produced in Lec1/Manic Fringe cells exhibited stronger inhibition than that produced in Lec1/vector cells, consistent with the fact that Fringe causes increased binding of Delta1 to Notch in ES cells and CHO cells. On the other hand, the Notch1 fragment with a T12A mutation produced in Lec1/vector cells did not inhibit Delta1-Fc binding to ES cells or to Lec1 CHO cells. Inhibition by the Notch1 T12A mutant fragment was not restored by producing it in Lec1/Manic Fringe cells. These results suggest that *O*-fucosylation on Notch EGF12 is critical for Delta1-Fc binding to mammalian Notch1.

(1) Shi, S. and Stanley, P. Proc. Natl. Acad. Sci. USA, 100, 5234-5239, 2003.
(2) Shao, L., Moloney, D., and Haltiwanger, R. J. Biol. Chem., 278, 7775-7782, 2003

Supported by NIH grant CA 95022 to P. S.

(350) **Forssman Glycolipid Expression During Differentiation of Mouse Embryonic Stem Cells and F9 Teratocarcinoma Cells**

Yasunori Kushi[1], Haruka Shinbo[1], Renuka karunagoda[1] and Shizuo Handa[2]
[1] Inada-cho, Obihiro-shi, Hokkaido, 080-8555, Japan, [2] Magome, Iwatsuki-shi, Saitama-ken, 339-8539, Japan.

Cell surface glycoconjugates are considered to behave as important key molecules in cell-cell and cell recognition. The change of the diverse strucutures are often associated with the differentiation event. Although Forssman glycolpid (FG) has been reported to be one of the heterophile antigens, this carbohydarate antigen is also remarkably changed during differentiation.To understand the glycolipid expression and the molecular mechanism, the expression of Forssman glycolipid was investigated in mouse F9 and ES cells. Both cells showed the high expression level in undifferentiated stage . However, this high expression level was decreased immediately after differentiation. In ES cells, the same carbohydarate expression was observed. The investigation of mRNA level of Forssman gkycolipid synthase exhibited the different expression patterns to that of cell surface carbohydrate antigen. The evidence was also confirmed by the glycolipid analysis on TLC, suggesting that there is other mechanism for regulation of glycolipid synthesis besides glycosyltransferase itself. Taken togather above,the expression of Forssman antigen did not correlate the FGSmRNA exression during differentiation, implicating that the regulation of Forssman antigen are controllled by many factors involved. Studies are in progress to address the factors detemining the decreaed FG expression in differentiated cells and biological consequences of the decline of FG expression containing preparation of the microdomain lipid fractions from F9 cells.

(351) **Differential Expression of Sialidases of Human Monocytes During Differentiation into Macrophages or Dendritic Cells**

Nicholas M. Stamatos[1,3], Feng Liang[2], Karine Landry[2], Alan S. Cross[3], Lai-Xi Wang[1] and Alexey V. Pshezhetsky[2]
[1] Institute of Human Virology, University of Maryland Medical System, Baltimore, MD, [2] Hopital Sainte-Justine and Departement de Pediatrie, University of Montreal, Montreal, Quebec, [3] Division of Infectious Diseases, Department of Medicine, University of Maryland Medical System, Baltimore, MD.

Sialidases are enzymes that influence cellular activity by removing terminal sialic acid from glycolipids and glycoproteins. Four genetically distinct sialidases have been identified in mammalian cells. We demonstrate that three of these sialidases, lysosomal Neu1 and Neu4 and plasma membrane-associated Neu3, are expressed in freshly-isolated, human monocytes. When measured using the artificial substrate 2`-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (4MU-NANA), sialidase activity increased as monocytes differentiated into macrophages or dendritic cells. This activity was attributable to Neu1, as greater than 99% of the activity was removed by immunoprecipitation of Neu1. In contrast, there was no reduction in the increased sialidase activity detected with mixed bovine gangliosides, suggesting that the expression of an additional sialidase was also increased during monocyte differentiation. Increases in Neu1- and Neu3-specific RNAs were detected by real time RT-PCR during monocyte differentiation. Likewise, western blot analysis with specific antibodies showed an increase in Neu1 and Neu3 proteins. During differentiation of monocytes, the lysosomal Neu1 translocated from its intracellular site to the cell surface as shown by immunohistochemistry. The functional significance of increased sialidase activity and cell surface expression of Neu1 during monocyte differentiation was studied in one model of microbial pathogenesis: HIV infection of monocytes/macrophages. Monocytes become permissive for growth of HIV-1 as they differentiate into macrophages or immature dendritic cells, coincident with the increase in sialidase activity. Using specific sialidase inhibitors, we show that infection of differentiated monocytes with HIV-1 can be blocked by inhibiting the endogenous sialidase activity. Thus, the differentiation of monocytes into macrophages and dendritic cells is associated with the specific regulation of distinct cellular sialidases, and the increase in sialidase activity during monocyte differentiation may play a role in the interaction of permissive cells with microbial pathogens.

(352) **Study of the Role of Sulfate Modification of Heparan Sulfate in Vivo and Screening for Compounds that Modify It.**

Sally E Stringer[1], Melissa A Rusch[2], Eleanor Y Chen[2], Stephen C Ekker[2] and Scott B Selleck[2,3]
[1] University of Manchester Department of Medicine, Manchester Royal Infirmary, Oxford Road, Manchester. M13 9WL. UK, [2] Department of Genetics, Cell Biology and Development, University of Minnesota, 6-160 Jackson Hall, 321 Church St SE, Mineapolis. MN 55455. USA
[3] Department of Pediatrics, University of Minnesota, Medical School. MMC 391, 420 Delaware St SE, Minneapolis. MN 55455. USA.

The biological role of sulfate modification of heparan sulfate in angiogenesis is not well understood *in vivo*. The feasibility of performing developmental, genetic and biochemistry studies in the zebrafish makes it an ideal system to address the biological role of sulfation modifications in a

vertebrate model system. We identified two zebrafish heparan sulfate 6-O-sulfotransferase (HS6ST) genes with dynamic, highly localized expression patterns during development. Study of HS6ST-2 morpholino antisense knockdown embryos demonstrated a unique role for 6-O sulfation in branching morphogenesis of the caudal vein plexus, a model for studying the complexity of vertebrate angiogenesis. HS6ST-1 did not appear to have a role in vascular development. Structural analysis of heparan sulfate from wildtype, HS6ST-1 and HS6ST-2 morphant embryos confirmed that there was a decrease in the level of 6-O-sulfated disaccharides in the morphant embryos. The distinct roles of HS6ST-1 and HS6ST-2 in embryonic development support the proposal that regulation of the sulfation pattern of heparan sulfate is a fine control mechanism of many physiological processes, from axon guidance to kidney development and that agents that alter heparan sulfate composition may be of therapeutic value. We are using a highly sensitive HPLC based method to screen for small molecules that affect heparan sulfate composition in a HepG2 liver carcinoma cell line. Initial screening shows that our approach is feasible and we have identified a number of leads that affect heparan sulfate structure in our tissue culture assay system.

(353) **Comparative Analysis of Oligosaccharides in Human Milk and Feces Using High Performance Thin Layer Chromatography**

David S. Newburg[1], Josef J. Polak III[1], Susan N. Wilhelm[2], Mary Beth Stepans[2], Mary S. Jacewicz[3] and Deborah S. Newburg[1]
[1] Program in Glycobiology, Mucosal Immunology Laboratory, Massachusetts General Hospital, Boston, MA 02129, [2] University of Nebraska College of Nursing, Scottsbluff, NE 69361, [3] Shriver Center of the University of Massachusetts Medical School, Waltham, MA 02452.

Oligosaccharides constitute the third largest solid component of human milk and are biologically active. Oligosaccharides are unique among major components of human milk in that they are mostly indigestible. The fecal oligosaccharide profile of breastfed infants resembles that of the milk they consume. A strong correspondence between oligosaccharide profiles from milk and from feces of breastfed infants indicates that much of the infant's dietary intake is human milk. The oligosaccharide profile of human milk varies by the genotype of the mother; the oligosaccharide profile of infant feces likewise resembles maternal milk phenotype. Screening populations of infants for insufficient intake of oligosaccharides, insufficiency of a specific oligosaccharide, and any relationship between oligosaccharide intake and health outcome would be facilitated by a simple and rapid technique for oligosaccharide profiling. The purpose of this study was to devise and evaluate a thin layer chromatographic method for comparing oligosaccharides in milk and feces of nursing dyads. Milk and feces samples were collected 2 weeks and 6weeks after parturition, with additional feces samples being collected after the infants were no longer breastfeeding at age 6 months. Crude oligosaccharides were extracted from 100 microliters of milk in 67% ethanol. Crude oligosaccharides were extracted from 100 milligrams feces by passing a fecal aqueous slurry (2 mL) through an anion-exchange column. Aliquots of oligosaccharides were separated by high performance thin layer chromatography (HPTLC) on a 200 micron layer of aminopropyl silica using a mobile phase consisting of pyridine: ethyl acetate: acetic acid: water (6:2:1:2, v/v/v/v). Oligosaccharides were visualized by spraying the plates with orcinol, 0.2% w/v in 2N sulfuric acid followed by heating. The plates were scanned electronically into digital files, and the image files were quantified with ONE-Dscan 2.2 on a Macintosh G3.† The performance characteristics of the method were evaluated using 3-fucosyllactose (3-FL) and lacto-*N*-pentaose II (LNF-II) as standards representative of human milk oligosaccharides.† The variance within TLC plates for 3-FL was 23 ± 13% (mean ± SD; n=13), and for LNF-II was 21 ± 16% (n=13). The coefficient of variation among plates was 33% for 3-FL (n=13) and 38% for LNF-II (n=13). The feces of breastfeeding infants had patterns of oligosaccharides that resembled the patterns of oligosaccharides of their mothers' milk; infants who were weaned had virtually no milk oligosaccharides in their feces.† Although HPTLC is often used qualitatively, this semi-quantitative application is inexpensive, rapid, and does not require expensive equipment, making it well suited for the large number of milk and fecal samples typical of a population study.

(354) **Characteristics of I β6-N-acetylglucosaminyltransferase-deficient Mice**

Guo-Yun Chen[1], Hisako Muramatsu[1], Mineo Kondo[2], Nobuyuki Kurosawa[1], Yozo Miyake[2], Naoki Takeda[3] and Takashi Muramatsu[1,4]
[1] Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya Japan, [2] Department of Ophthalmology, Nagoya University School of Medicine, Nagoya, Japan, [3] Center for Animal Resources and Development, Kumamoto University, Kumamoto, Japan, [4] Department of Health Science, Faculty of Psychological and Physical Sciences, Aichi Gakuin University, Nisshin, Aichi, Japan.

Poly-N-acetyllactosamines are glycans with repeated Galβ1-4GlcNAcβ1-3 units. Branching of poly-N-acetyllactosamines is catalyzed by a specific β6-N-acetylglucosaminyltransferase, designated as I β6-N-acetylglucosaminyltransferase (IGnT) based on the fact that branched poly-N-acetyllactosamines bear blood group I antigen. Erythrocyte poly-N-acetyllactosamines carry ABH blood group antigens and embryonal poly-N-acetyllactosamines carry LeX antigen. Expression of branched poly-N-acetyllactosamines is developmentally regulated. They are abundantly expressed during early embryogenesis, and this expression progressively decreases during development. Polyvalent ligands expressed on the branched structure are expected to have stronger binding activity as demonstrated in an artificial selectin ligand. To gain overall picture of the functional significance of the branching, we produced mice deficient in the IGnT gene. In both humans and mice, three molecular species of IGnTs have been identified, and a common exon is present in all of them. We deleted the common exon to knockout all the IGnTs. The deficient mice were born, developed and reproduced normally. IGnT activity was abolished in the stomach, kidney, bone marrow and cerebellum of the deficient mice, while a low level of the activity persisted in the small intestine. Immunohistochemical analysis confirmed the loss of IGnT activity in the lung, kidney and stomach. We found that spontaneous locomotive activity, the number of lymphocytes in the blood and renal function were reduced in the deficient mice. The results suggest the importance of poly-N-acetyllactosamine branching in function of certain tissues. On the other hand, cataracts did not develop earlier in the deficient mice, although development of cataracts has been correlated with the loss of function of the common exon in humans.

(355) **Effects of Immunomodulatory Drugs on Galectin-3**

Sanja DabeliÊ, Jerka DumiÊ and Mirna Flˆgel
Department of Biochemistry and Molecular Biology, Faculty of Pharmacy and Biochemistry, University of Zagreb,
A. Kova'iÊa 1, 10000 Zagreb, Croatia.

Galectin-3, a 29-32 kDa β-galactoside binding lectin, is implicated in many biological processes, (e.g. growth regulation, pre-mRNA splicing, cell differentiation, neoplastic transformation and tumor metastasis). It plays a key role in immune responses by modulating T-cell apoptosis, proliferation, cell adhesion, chemotaxis and synthesis of inflammatory mediators such as cytokines, nitric oxide and prostaglandines. Galectin-3 expression is influenced by various stimuli but the precise regulatory mechanisms are not yet elucidated. In our previous studies we showed the involvement of the NF-kappaB and AP-1 transcription factors in regulation of galectin-3 expression. It was also shown that galectin-3 expression in macrophages is regulated by Ras/MAP kinase signalling pathway. The mentioned transcription factors and signalling pathway are strongly influenced by many anti-inflammatory drugs. Therefore, the aim of our study was to determine the effects of steroid (hydrocortisone - HC and dexamethasone - Dex) as well as non-steroid immunomodulatory drugs (aspirin - Asp and indomethacin - Ind) on the expression of galectin-3 both on the mRNA and protein level. The human monocytic cell line THP-1 was exposed to various concentrations of Asp (0.1, 0.5, 1 and 2 mM), Ind (0.1, 1, 5 and 10 microM), HC (0.1, 1, 5 and 10 microM) and Dex (0.1, 1, 10 and 100 nM) during 1, 3, 5, 24, 48 or 72 hrs. The target gene mRNA level is evaluated using relative RT-PCR method and GeneScan analysis software. The results showed that all concentrations of applied drugs halved the galectin-3 mRNA level already after 1 h of exposure (except 0.1 mM aspirin which showed that effect after 24 h) and that the level of mRNA continued to decrease during time and was only 4-30% of the initial value after 72 h. Surprisingly, the protein expression measured by chemiluminescent-western blot analysis didn't follow the same pattern - the decrease was observed only after 48 and 72 h of exposure, when the galectin-3 level fall on 70% and 50% of the initial value, respectively.

(356) **Molecular Interaction Between Fetuin and 15 Phenolic Compouds, Including Environmental Hormones by SPR Analysis.**

Naoko Masuda, Yasuhiro Ozeki and Shinji Asakura
Facul. of Sci, Dept.of Biochem.Yokohama City Univ. 22-2,Seto,Kanazawaku,Yokohama JAPAN 236-0027.

Endocrine system consists of hormone production, target and transport environments. .Many part of steroid and thyroid hormones are transported with hormone binding proteins, such as sex-hormone binding protein and albumin, in sera.Fetuin is a 43kDa serum glycoprotein which increases in fetus and acute phase of mammal. As the biological activity, fetuin was reported to have the stimulation of lymphocyte, promotion of cell growth, and trypsin inhibition. But the physiological function of the protein is unclear yet. Using a surface plasmon resonance (SPR) biosensor, we analyzed the interactions between bovine fetuin and fifteen phenolic compounds such as estrogen and flavone. The binding activity of each 30É M of such chemicals as environmental hormone(4-nonylphenol and bisphenol A), synthetic hormone, (diethylstilbestrol), strogen antagonist drug, (tamoxfen), polyphenol, (catechin), soy bean isoflavone (genistein), with a fetuin-fixed sensor chip was tested. Chemicals appeared different dissociation constants between e-3M(cholesterol) and e-5M(environmental hormone, polyphenol, and isoflavone). Some drugs such as warfarin appeared binding to fetuin with dissociation constant at e-4M. This value was similar with that of dissociation constant of the drug to bovine serum albumin. This analysis suggested that a glycoprotein fetuin is possible to function making the environment for the transportation of steroid hormone and phenolic compounds as same as albumin.

(357) **Tnf Alpha-Induced Insulin Resistance in Adipocytes as a Membrane Microdomain Disorder:Involvement of Ganglioside Gm3**

Kazuya Kabayama[1,2], Takashige Sato[1], Futoshi Kitamura[1], Satoshi Uemura[1], Byoung Won Kang[1], Yasuyuki Igarashi[1] and Jin-ichi Inokuchi[1,2]

[1] 1Department of Biomembrane and Biofunctional Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, [2] Core Research for Evaluational Science and Technology program (CREST), Japan Science and Technology Corporation (JST).

Membrane microdomains (lipid rafts) are now recognized as critical for proper compartmentalization of insulin signaling, but their role in the pathogenesis of insulin resistance has not been investigated. Detergent-resistant membrane microdomains (DRMs), isolated in the low density fractions, are highly enriched in cholesterol, glycosphingolipids and various signaling molecules. TNF α induces insulin resistance in type 2 diabetes, but its mechanism of action is not fully understood. We have found a selective increase in the acidic glycosphingolipid ganglioside GM3 in 3T3-L1 adipocytes treated with TNF α, suggesting a specific function for GM3 (Tagami S., Inokuchi J., Kabayama K., Yoshimura H., Uemura S., Ogawa C., Ishii A., Saito M., Ohtsuka Y., Sakaue S., and Igarashi Y., *J. Biol. Chem.*, 277, 3085-3092, 2002). In the DRMs from TNF α-treated 3T3-L1 adipocytes, GM3 levels were doubled, compare to results in normal adipocytes. Additionally, insulin receptor (IR) accumulations in the DRMs were diminished, while caveolin and flotillin levels were unchanged. Furthermore, insulin-dependent IR internalization and intracellular movement of the IR substrate 1(IRS-1) were both greatly suppressed in the treated cells, leading to an uncoupling of IR-IRS-1 signaling. GM3 depletion was able to counteract the TNF α-induced inhibition, of both the IR internalization and its elimination from the DRMs. Together, these findings provide compelling evidence that in insulin resistance the insulin metabolic signaling defect can be attributed to a loss of IRs in the microdomains due to an accumulation of GM3.

(358) **Molecular Mechanism and Evolutional Analysis of Human Influenza A Virus N2 Neuraminidase Genes Based on the Transition of the Low-Ph Stability of Sialidase Activity**

Tadanobu Takahashi[1], Takashi Suzuki[1], Takehiko Saito[2], Chao-Tan Guo[1,3], Kazuya I.-P. Jwa Hidari[1], Daisei Miyamoto[1] and Yasuo Suzuki[1]

[1] Department of Biochemistry, University of Shizuoka, School of Pharmaceutical Sciences, CREST, JST, and COE Program in the 21st century, Shizuoka 422-8526, Japan, [2] Department of Virology III, National Institute of Infectious Diseases, Musashi-Murayama 208-0011, Tokyo, Japan, [3] Institute of Bioengineering, Zhejiang Academy of Medical Sciences, 182 Tianmushan Road, Hang Zhou 310013, China.

New pandemic influenza A viruses seem to be generated by transmission of avian viruses to other animal species or by genetic reassortment between avian and other host viruses. The H2 and H3 subtypes of hemagglutinin (HA) appeared in the 1957 and 1968 pandemic strains by genetic reassortment from the avian influenza viruses, while the N2 subtype of neuraminidase (NA) first appeared in the 1957 strain and was inherited in the 1968 pandemic strain from the prevalent H2N2 virus in humans. With regard to the evolution of N2 NAs in humans, NA genes of H3N2 viruses isolated between 1968 and 1997 have been shown to share essentially a single lineage with short side branches; however, little is known about the properties of the original strain from which the NA gene of the 1968 pandemic influenza virus was introduced. We have also found that the 1957 and 1968 human pandemic influenza A viruses as well as duck viruses retained their sialidase activities even at pH of less than 4.5 but that some of the human H2N2 epidemic strains isolated before 1968 and human epidemic H3N2 strains isolated after 1968 had no sialidase activities under the same conditions. These findings indicate that low-pH stability of influenza A virus sialidase activity may be a critical factor for viral replication in ducks and may be an important point for determining the epidemiology of human influenza A viruses. In this study, we examined the low-pH stabilities of sialidase activities of cell-expressed N2 NAs by using chimeric NAs and site-specific mutagenesis, and we found that consensus amino acid regions responsible for low-pH stability did not exit in pandemic NAs but that two amino acid substitutions in the low-pH-stable A/Hong Kong/1/68 (H3N2) and a single substitution in the low-pH-unstable A/Texas/68 (H2N2) NA resulted in significant change in the low-pH stability. We therefore generated mutant influenza A viruses that included N2 NA genes, in which pH stability of the sialidase activities were changed, by using plasmid-driven reverse genetics system. The NA mutant viruses showed a significant difference in viral infection. We also investigated the transition of avian (duck)-like low-pH stability of sialidase activities with the evolution of N2 NA genes in human influenza A virus strains. We found that the NA genes of H3N2 viruses isolated from 1971 to 1982 had evolved from the side branches of NA genes of H2N2 epidemic strains isolated in 1968 that were characterized by the low-pH-unstable sialidase activities, though the NA genes of the 1968 pandemic strains preserved the low-pH-stable sialidase. These findings suggest that the prototype of the H3N2 epidemic influenza strains isolated after 1968 probably acquired the NA gene from the H2N2 low-pH-unstable sialidase strain by second genetic reassortment in humans.

(359) **Beta-Glucan "Pro-Drugs": Large Beta-Glucans are Processed by Macrophages that Secrete a Bioactive Fragment that Primes Neutrophils to Kill Antibody-Targeted Tumor Cells**

Gordon D. Ross[1], Daniel J. Allendorf[1], Richard D. Hansen[1], Jun Yan[1] and Brian K. Brandley[2]

[1] Tumor Immunobiology Program, James Graham Brown Cancer Center, University of Louisville, Louisville, KY,

[2] Pharmaceutical Division, Biopolymer Engineering, Inc., Eagan, MN.

Beta-Glucans have been shown to function against cancer by priming the complement C3-receptor of blood neutrophils, CR3, to mediate cytotoxicity of tumors coated with the C3 fragment iC3b. Specific targeting of tumors is accomplished with anti-tumor monoclonal antibodies (mAb) that bind to tumors and activate the complement system, thereby depositing the iC3b target on tumor cells. Intravenous (i.v.) therapy with small (10 kD) neutral soluble b-glucan, a single-chain b(1,3;1,6)glucan isolated from baker's yeast, results in priming the CR3 of circulating neutrophils. Another therapeutic b-glucan consists of whole b-glucan particles (EX-WGP) that are given orally. EX-WGP are taken up by gastrointestinal macrophages that shuttle them to bone marrow where they degrade EX-WGP into soluble NSG-like fragments that prime the CR3 of marrow neutrophils. In mouse tumor models, PGG, a large (140 kD) soluble b-glucan, mediated both tumor regression and long-term survival when used in combination with a complement-activating anti-tumor mAb. In vitro studies showed that macrophages degraded PGG into small fragments capable of optimally priming neutrophil CR3. Because i.v. PGG is primarily taken up by liver macrophages (Kupffer cells), it appears likely that i.v. PGG is similarly degraded by Kupffer cells into small b-glucan fragments that prime the CR3 of neutrophils circulating through the liver. The time required for this Kupffer cell processing allowed a single i.v. dose given weekly to mediate better tumor regression than did i.v. NSG given daily. Weekly i.v. injections of PGG were equivalent to oral EX-WGP given daily, while mice given both i.v. PGG weekly and daily oral EX-WGP exhibited significantly better tumor regression and survival. Weekly i.v. PGG could be co-administered with therapeutic mAbs such as Herceptinô and Rituxanô that are also given by weekly i.v. infusion. Most patients could also take oral EX-WGP for enhanced tumor regression.

(360) **The Bioavailability and Pharmacokinetics of Chondrosine After Oral and Intravenous Single Dose Administration in Mice**

Shuichi KUSANO[1], Naoko IGARASHI[2], Shinobu SAKAI[2], Hidenao TOYODA[2] and Toshihiko TOIDA[2]

[1] The Research Institute of Fuji Sangyo Co. Ltd., Marugame, Kagawa,

JAPAN,
[2] Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, JAPAN.

OBJECTIVE: The purpose of this study was to determine if chondrosine (CSdi) is absorbed after oral administration to mice by the new analytical HPLC method for CSdi. The bioavailability of CSdi was evaluated by the direct measurement of CSdi found in the plasma following a new pretreatment procedure using a spin tube with a molecular weight (5,000 Da) cut-off filter membrane. METHODS: The postcolumn HPLC method for the determination of CSdi was performed on an SCX column (4.6 mm i.d. x 150 mm), 50 mM borate buffer (pH 4.6) as an eluent (0.8 mL/min), 1% 2-cyanoacetamide and 1.0 M NaOH as fluorogenic reagents (0.25 mL/min each) with a fluorescence detector (Ex. 331 nm, Em. 383 nm). The fluorogenic reaction was carried out in a reaction coil (0.5 mm i.d. x 10 m) at 110Åé. Two separate animal studies were conducted. In study 1, adult female mice (N=35) received the following treatment: i.v. CSdi (5 mg/kg body weight) and the plasma samples were collected at 0, 5, 15, 30, 60, 120 and 180 min after the administration of CSdi. In a second study, 30 adult female mice received the following treatment: p.o. CSdi (400 mg/kg body weight) and the plasma samples were collected at 30, 60, 120, 180, 240 and 300 min after the oral administration of CSdi. Blood plasma samples were de-proteinized, analyzed and pharmacokinetic parameters were determined. The pretreatment procedure was newly developed for the microdetermination of CSdi in blood plasma using a spin tube with molecular weight (5,000 Da) cut-off filter membrane. Fifty microliters of blood plasma is required for the assay and galactosamine hydrochloride was added as an internal standard before the pretreatment. RESULTS: CSdi was absorbed after oral administration with a mean C(max) of 12.5 microgram/mL and a mean T(max) of 3.0 h. CONCLUSIONS: This study provides the first report of the bioavailability of orally administered CSdi in mice. CSdi is expected to stimulate the biosynthesis of chondroitin sulfate and the effect of chondrosine on chondroitin sulfate biosynthesis in the cell line induced from bovine cartilage is under investigation.

(361) **Endogenously Produced Ganglioside GM3 Endows Anticancer Drug Resistance Phenotype By Upregulating Bcl-2 Expression in 3LL Lewis Lung Carcinoma Cells**

Mariko Noguchi[1], Kazuya Kabayama[1,2], Satoshi Uemura[1], Byoung Won Kang[1], Masaki Saito[3], Yasuyuki Igarashi[1] and Jin-ichi inokuchi[1,2]
[1] Department of Biomenbrane and Biofunctional Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University,
[2] Core research for evaluational science and technology program (CREST), Japan science and technology corporation (JST),
[3] Pharmacodynamics, Meiji Pharmaceutical University.

The relationship between gangliosides and cancer malignancy has long been focused because the ganglioside patterns have dramatically changed during cell differentiation and cancer malignancy. To investigate the significance of ganglioside GM3 in cancer malignancy, we established ganglioside GM3-reconstituted cells by transfecting cDNA of GM3 synthase (SAT-I) into the GM3-defficient J5 subclone of 3LL Lewis lung carcinoma cells (Uemura, S., Kabayama, K., Noguchi, M., Igarashi, Y., and Inokuchi, J. [2003] Glycobiology 13, 207-216). The GM3-reconstituted cells were resistant to apoptosis induced by etoposide and doxorubicin. There were no changes in the expression levels of topoisomerase IIα and P-glycoprotein, as well as the uptake of doxorubicin between the GM3-reconstituted cells and the mock cells. To reveal the mechanism of the etoposide-resistant phenotype acquired in the GM3-reconstituted cells, we investigated apoptotic signaling by exposing SAT-I transfectants to two distinct pro-apoptotic agents, TNF-α and etoposide. TNF-α plus cycloheximide stimulate a cell death receptors and etoposide utilizes the mitochondrial apoptosis pathway. TNF-α plus cycloheximide induced DNA fragmentation in SAT-I transfectants and mock cells, however, etoposide treatment didn't induce DNA degradation in SAT-I transfectants. These results imply that GM3-reconstituted cells show resistant to apoptosis mainly in the mitochondrial pathway. Next, we investigated the mitochondrial apoptosis pathway in detail. Although no difference was observed in the phosphorylation of p53 at Ser15 site by etoposide between the GM3-reconstituted cells and mock cells, the activations of caspase-3 and caspase-9 by etoposide were specifically inhibited in the former. We found that anti-apoptotic protein Bcl-2 was increased in the GM3-reconstituted cells. Moreover, wild type 3LL Lewis lung carcinoma cells which express GM3 rich didn't show DNA fragmentation induced by etoposide and expressed higher level of Bcl-2 protein compared with the J5 subclone. Thus, these results support that endogenously produced GM3 positively involves in malignant phenotypes including anti-cancer drug resistance, probably through upregulating Bcl-2 protein.

(362) **Increased Fucosylation and Branching of Serum Tranferrin N-Glycans in Long-Term Untreated Galactosemic Patients.**

Luisa Sturiale[1], Rita Barone[2], Giovanni Sorge[2], Marco Zaffanello[3], Agata Fiumara[2], Giuseppe Impallomeni[1] and Domenico Garozzo[1]
[1] Istituto di Chimica e Tecnologia dei Polimeri -CNR - Viale R. Margherita 6, 95123 Catania, Italy,
[2] Centro per le Malattie Metaboliche Ereditarie - Dipartimento di Pediatria - Universit‡ di Catania - Via S. Sofia 78, 95125 Catania, Italy,
[3] Dipartimento di Pediatria - Universit‡ di Verona - Piazzale L. Scuro, 37134 Verona, Italy.

Galactosemia is an autosomal recessive disorder caused, in the most common form, by mutation in the galactose-1-phosphate uridyltransferase (GALT) gene (ch. 9p13). The resulting enzyme deficiency leads to anomalous accumulation of galactose and gatactose-1-phosphate in blood and tissues and produces severe symptoms as mental retardation, cirrhosis of the liver and cataracts, prevented by a galactose-free diet. In untreated galactosemic patients, isoform patterns of serum transferrin, lysosomal enzymes β-hexosaminidase and α-fucosidase and follicle stimulating hormone are abnormal, due to the increase of relatively neutral isoforms corresponding to less sialylated carbohydrate structures [1-2]. These evidences are similar to those observed in the Congenital Disorders of Glycosylation (CDG) which are inherited disorders characterized by a defective synthesis of the carbohydrate moiety of multiple serum glycoproteins. In order to investigate the glycosylation abnormalities in galactosemic patients, we used MALDI mass spectrometry to individuate the glycosylation degree of intact glycoproteins and to achieve the fully characterization of the N-linked oligosaccharide structures. Particularly, we focused on the glycosylation pattern of rivanol purified serum transferrin (the classical biochemical marker of CDGs) in two galactosemia patients before treatment and during follow-up on galactose-free diet. The results were compared with those obtained in subjects with CDG-Ia (PMM deficiency) and healthy controls. The galactosemic patients were both overexposed to dietary galactose (11 and 5 weeks respectively) because of an initial false negative newborn screening following red blood cell transfusion. In long-term untreated galactosemia we found a severe underoccupancy of both transferrin N-glycosylation sites according to the isoelectric focusing pattern and the profile of MALDI-TOF mass spectra of the intact glycoprotein. Release of N-glycans after PNGase F digestion of the same samples, followed by MALDI-TOF analysis both in negative and in positive polarity, revealed a great heterogeneity of glycoforms ranging from truncated biantennary species deprived of sialic acid and/or galactose, to triantennary and tetraantennary species at higher molecular weight. Moreover, we found a significant increase of the fucosylation degree of all the glycoforms, already reported for CDG-I [3], but never observed before in galactosemia. Such abnormal findings were not observed upon dietary treatment. These evidences suggest that in long-term untreated galactosemia, defects in both the assembly as well as in the processing pathways may occur. These findings open the way to a better comprehension of the biochemical mechanisms regarding the defective glycosylation pathway in galactosemia. References: 1. Jaeken J, Kint J, Spaapen L. Lancet 1992; 340: 1472-1473. 2. Charlwood J, Clayton P, Keir G, Mian N, Winchester B. Glycobiology 1998; 4: 351-357. 3. Mills P, Mills K, Clayton P, Johnson A, Whitehous B. Biochem J 2001; 359: 249-254.

(363) **Carbohydrate Phenotyping of Human and Animal Milk Glycoproteins: the Use of Pig Milk as an Inhibitor of Helicobacter Pylori Colonization**

Anki Gustafsson[1,5], Anna Hultberg[5], Rolf Sjˆstrˆm[3], Imre Kacskovics[4], Michael E Breimer[2], Thomas BorÈn[3], Lennart Hammarstrˆm[5] and Jan Holgersson[5]
[1] Department of Clinical Chemistry, Sahlgrenska University Hospital, Gˆteborg, [2] Department of Surgery, Sahlgrenska University Hospital, Gˆteborg, [3] Department of Medical Biochemistry and Biophysics, UmeÂ University, UmeÅ,
[4] Department of Physiology and Biochemistry, Faculty of Veterinary Science, Szent Istv·n University, Budapest,
[5] Division of Clinical Immunology, Karolinska Institutet, Karolinska University Hospital at Huddinge, Stockholm.

Breast-milk has a well-known anti-microbial effect, which is in part due to the many different carbohydrate structures expressed. This renders it a position as a potential therapeutic for treatment of infections caused by

different pathogens, thus avoiding the drawbacks of many antibiotics. The repertoire of carbohydrate epitopes in breast-milk is known to differ between species, with human milk expressing the most complex one. We have investigated the expression of protein-bound carbohydrate epitopes in milk from man, cow, goat, sheep, pig, horse, dromedary and rabbit. The precursor chains of several carbohydrate epitopes known to mediate binding of pathogens were found in milk from all animals investigated. Blood group ABH, Le^x, sialyl-Le^x, Le^a, sialyl-Le^a and Le^b epitopes were mainly expressed in milk from man, pig and to some extent horse, whereas milk from the other species expressed very low levels of these epitopes. Interestingly, both man and horse milk proteins exhibited anti-pk reactivity. Expression of the H. pylori receptors, sialyl-Le^x and Le^b, on porcine milk proteins was breed- and individual-specific, and correlated to the ability of porcine milk to inhibit H. pylori adhesion in vitro and H. pylori colonization in vivo. Thus, milk from certain pig breeds may have a therapeutic and/or prophylactic effect on H. pylori colonization.

(364) **GM3-Binding Peptides Inhibit Influenza Virus Infection**

Teruhiko Matsubara[1], Machiko Sumi[1], Takao Taki[2] and Toshinori Sato[1]
[1] Department of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan, [2] Molecular Medical Science Institute, Otsuka Pharmaceutical Co. Ltd., 463-10 Kagasuno, Kawauchi, Tokushima 771-0192, Japan.

Sialyloligosaccharides on the cell surface have been known as receptors for pathogenic molecules and as molecular markers for tumor cell. Hemagglutinin of influenza virus recognizes α2-3 and α2-6-linked of sialylgalactose structures. If we can design the molecules that inhibit the interaction between hemagglutinin and sugar on the cells, the inhibitor would be a candidate of medicine for influenza virus. Many inhibitors such as sugar-modified polymers and liposomes have been designed. We identified pentadecamer (15-mer) peptides that bind to sugar chain containing *N*-acetylneuraminic acid (Neu5Ac) by the selection of a phage-displayed random peptide library. An affinity selection was performed against a lipid monolayer of ganglioside GM3 (Neu5Acα2-3Galβ1-4Glcβ1-1'Cer). Seven kinds of GM3-binding phage clones were isolated and the interactions between the phage clones and animal cells were investigated. A phage clone had affinity for B16 melanoma cells at 10 nM of phage concentration. To confirm that the binding of phage is mediated by sialic acid on the cell surface, inhibition assay and neuraminidase treatment of cells were performed. The binding of phage clone was inhibited with 1 mM of sialic acid, and no inhibition by glucose was found. After the removal of sialyl residues of glycoprotein by the neuraminidase, the phage lost the affinity for the cells. These results indicated that the selected phage bound to Neu5Ac-containing sugar chain on the cells. Two synthetic pentadecapeptides, c01 (GWWYKGRARPVSAVA) and c03 (RAVWRHSVATPSHSV), were chemically synthesized and the binding affinities for glycolipids were determined by quartz-crystal microbalance method. These peptides showed high affinity of c01 and c03 for Neu5Acα2-3Gal with dissociation constant of 9.0 and 4.5 micromol/L, respectively. Then *N*-stearoyl derivatives of the c01 and c03 peptides were prepared to incorporate into liposome. In the presence of the peptide-containing liposomes, no infection of influenza A virus (H1N1) to Madin-Darby canine kidney (MDCK) cells were observed. Since there are Neu5Acα2-3Gal and Neu5Acα2-6Gal structures on the MDCK cells, it is considered that binding of the liposomes to the cell surface resulted in the inhibition of the influenza virus. We showed that the phage clone specifically bound to the sialyl group of glycoconjugates on the cell membrane and the synthetic peptides inhibited the infection of influenza virus to host cells. Many sugar-binding proteins such as lectins and antibodies have been utilized to label glycoconjugates and applied to therapy for carbohydrate-related diseases. Our results suggested that the selected peptides have the possibility for the medical application as well as the sugar-binding proteins.

(365) **Directed Evolution of Sugar-Replica Peptide Having Hemagglutinin-Binding Ability**

Ai Onishi, Teruhiko Matsubara and Toshinori Sato
Department of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan.

Influenza A virus is a causative agent of serious disease for a long time, because there has been little development of effective anti-influenza virus agent. The virus have the capacity to modify its surface antigens of hemagglutinin (HA) and sialidase (NA), which leave the immune system unable to cope with new antigens. The X-ray crystallographic data have helped us to design improved inhibitors against the target HA and NA of the virus. Influenza HA is known to bind sialylgalactoside having α2-3- or α2-6- linkages on host cells at the first step of infection. Some sialic acid-containing polymers have been designed for inhibition of virus infection, but these polymers might be digested by NA. Therefore, we attempted to design peptides as universal inhibitors that bind to the receptor-binding pocket of HA. In order to obtain peptides which are a mimic of sialyl oligosaccharide, a phage-displayed random pentadecapeptide library have been employed in our study. The phage-displayed method is a selection technology using a pool of phage in which each virion expresses a different peptide sequence as a part of its coat protein. It was expected that HA-binding peptides selected from the phage library would serve as inhibitors of influenza A virus. Our previous work showed that the HA-binding peptide, ARLSPTMVHPNGAQP (A-1), inhibited the infection of both H1 and H3 subtypes of influenza virus to Mardin-Darby canine kidney cells. Although the theoretical molecular diversity of pentadecapeptide is calculated to be $3.3x10^{19}$, the phage library employed in our previous study has only $2.5x10^8$ kinds of diversity. Therefore, improved peptide sequences are expected to be found out by sublibrary generated from a selected sequence. In the present study, we attempted the directed evolution approach to improve the binding affinity of the A-1 sequence. An error-prone PCR was used to generate a sublibrary containing mutation of one or two amino acid per 15-mer peptide. The error-prone PCR is a strategy for introducing random mutations enzymatically by polymerases. Another sublibrary of A-1 was also constructed by incorporating a synthetic oligonucleotide designed to generate the mutation of five amino acids per 15-mer peptide. The two sublibraries were employed to obtain HA-binding peptides. H1 and H3 were extracted with ether from influenza virus A/New Caledonia/20/99 and A/Panama/2007/99, respectively. Two affinity selections using the sublibraries were performed alternately against H1 and H3. Then the phages bound to HA were eluted by sialyl Lewis X. After 4 or 6 rounds of selections, several peptide sequences having mutation of 3-4 amino acids were obtained. The binding specificities of the isolated phages against HA were determined by ELISA. Many of the mutant phage clones showed higher binding affinity for both H1 and H3 subtypes than the original A-1 phage.

(366) **4-Methylumbelliferon Inhibits Metastasis of Melanoma Cells.**

Atsushi Kon[1], Shuichi Yoshihara[2], Daisuke Kudo[1], Ikuko Kakizaki[1], Ryousuke Taniguchi[1], Masahiko Endo[1] and Keiichi Takagaki[1]
[1] Department of Biochemistry, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan, [2] Department of Surgery, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan.

Hyaluronan (HA) is a non-sulfated glycosaminoglycan of high molecular mass, which consists of repeated β-1,4-GlcUA-β-1,3-GlcNAc disaccharide units. It is present in various kinds of tissues as a major component of the extracellular matrix and plays an important role in tissue organization, wound healing and inflammation. Several studies have demonstrated that HA participates in various phases of tumor metastasis, i.e., in cell adhesion and locomotion. HA overproduction by malignant tumor cells enhances their metastatic potential and leads to a worse clinical prognosis, suggesting HA is a risk factor for tumor metastasis. We have previously reported that 4-methylumbelliferone (MU) inhibits HA synthesis and may be not only a useful tool for examining the functions of HA but also an inhibitor of tumor metastasis. In this study, we have investigated whether MU inhibits the metastasis of melanoma cells that form malignant tumors with high metastatic potential. The formation of cell surface HA by cultured B16-F10 melanoma cells, and release of HA into the culture medium, are inhibited by MU. Adhesion and locomotion assays revealed that the adhesion and locomotion were dose-dependently inhibited by MU. Conversely, treatment with exogenous HA enhanced both adhesion and locomotion. Thus, preventing the formation of cell surface HA reduced both the adhesion and locomotion of melanoma cells, suggesting that MU may act as an inhibitor of tumor metastasis. Finally, we demonstrated that oral administration of MU reduced both HA production in the liver of C57BL/6 mice and the formation of metastatic nodules when melanoma cells are injected into the lateral tail vein *in vivo*. Furthermore, formation of metastatic nodules is also decreased by injection of melanoma cells pre-treated with MU. Thus, reduction of both melanoma cell surface HA and host liver HA inhibit metastasis. Therefore, MU is not only a useful tool for investigating the various functions of HA but also has potential as a novel therapeutic agent that controls the metastasis of melanoma cells.

(367) **GlcNAc 6-*O*-sulfotransferase-2 Ectopically Expressed in Ovarian Mucinous and Clear Cell Carcinoma Cells**

Akira Kanoh[1], Akira Seko[1,2], Masaru Sakamoto[3] and Katsuko Yamashita[1]
[1] Dept. of Biochem., Sasaki Institute, [2] CREST, [3] Kyoundo Hospital.

GlcNAc6ST-2 is a member of the Gal / GalNAc / GlcNAc 6-*O*-sulfotransferase family, and it is involved in the biosynthesis of a L-selectin ligand. We previously reported that GlcNAc6ST-2 is ectopically expressed in colonic mucinous adenocarcinomas and not in colonic non-mucinous adenocarcinomas or normal colon epithelia (Seko et al. *Glycobiology* 12, 379, 2002). We aimed to elucidate whether GlcNAc6ST-2 is ectopically expressed in mucinous carcinomas derived from other organs. In this report, we investigated its expression in ovarian carcinoma. Surgically derived paraffin tissue sections of 41 ovarian tumors were immunohistochemically tested using with antisera against GlcNAc6ST-2. As a result, all specimens derived from ovarian mucinous adenocarcinomas expressed GlcNAc6ST-2 (10 positives / 10 samples). Clear cell adenocarcinomas also expressed GlcNAc6ST-2 at a high rate (6 / 9), whereas endometrioid adenocarcinomas (1 / 7) and serous adenocarinomas (3 / 10) expressed it at a low rate. Mucinous benign adenomas were not stained by GlcNAc6ST-2 antisera (0 / 5), indicationg that its expression is malignancy-specific manner. This was confirmed by semi-quantitative RT-PCR analysis, which showed that the mucinous adenocarcinomas expressed GlcNAc6ST-2 transcripts at high level, whereas mucinous benign adenomas expressed it at low level. HID staining indicated that mucinous and clear cell adenocarcinomas produce sulfated glycans. These results suggested that GlcNAc6ST-2 is ectopically expressed in mucinous and clear cell adenocarcinomas of ovarian tumors, and that the enzyme may be responsible for sulfomucin biosynthesis in these tumor tissues. Ovarian mucinous and clear cell carcinomas are known to be resistant against cisplatin-based chemotherapy, and caused worse prognosis. We are studying whether sulfoglycans synthesized by GlcNAc6ST-2 in ovarian carcinoma cells are involved in the chemoresistance.

(368) **Differeces in Receptor Specificity of Human and Murine Respiroviruses**

Takashi Suzuki[1], Toru Takimoto[2], Allen Portner[3], Yoko Akashi[1], Tomomi Suzuki[1], Chika Hosokawa[1], Hiroo Ueyama[1], Tadanobu Takahashi[1], Chao-Tan Guo[1], Kazuya I.-P. Jwa Hidari[1], Daisei Miyamoto[1] and Yasuo Suzuki[1]
[1] University of Shizuoka School of Pharmaceutical Sciences, Department of Biochemistry, CREST JST, and COE Program in the 21st Century, Shizuoka, Japan, [2] University of Rochester, Medical Center, Department of Microbiology and Immunology, Rochester, United States, [3] St. Jude Children's Research Hospital, Division of Virology Department of Infectious Diseases, Memphis, United States.

Human parainfluenza viruses are important respiratory tract pathogens. The human parainfluenza virus type 1 (hPIV-1) causes most cases of laryngotracheobronchitis (croup) in children, and the human parainfluenza virus type 3 (hPIV-3) is second only to respiratory syncytial virus as a cause of pneumonia and bronchiolitis in infants less than 6 month of age. These viruses, which belong to the genus Respirovirus and the family Paramyxoviridae, have two spike glycoproteins, the hemagglutinin-neuraminidase (HN) glycoprotein and the fusion (F) glycoprotein, embedded in the envelope. Through its hemagglutinin-neuraminidase glycoprotein, parainfluenza viruses bind to sialic acid-containing glycoconjugates to initiate infection. Among the parainfluenza viruses, only Sendai virus (SV), the murine respirovirus, has been characterized in detail for its receptor determinants in several model systems. Although the virus-receptor interaction is a key factor of infection, the exact nature of the receptors that human respiroviruses recognize has not been determined. We determined the abilities of human respiroviruses to bind to different types of gangliosides. We found that the receptor specificity of parainfluenza viruses varies among subtypes and that the core structure of the sugar chain constitutes an important part of the receptor recognized by hPIV-1 and hPIV-3. Both hPIV-1 and hPIV-3 preferentially bound to neolacto-series branched gangliosides containing a terminal *N*-acetyl neuraminic acid (NeuAc) linked to *N*-acetyllactosamine by the α2-3 linkage. Unlike hPIV-1, hPIV-3 bound to neolacto-series gangliosides with terminal NeuAc linked to *N*-acetyllactosamine through α2-6 linkage or to the gangliosides with a terminal *N*-glycolyl neuraminic acid (NeuGc). To determine whether preferential binding to oligosaccharides containing branched *N*-acetyllactosaminoglycans with terminal NeuAcα2-3Gal is the general character of human respiroviruses, we evaluated binding specificities of human respiroviruses to six kinds of sialylglycoproteins with known terminal carbohydrate sequences by solid-phase binding assay. hPIV-1 and hPIV-3, as well as SV, showed the high binding ability for bovine erythrocyte membrane sialylglycoprotein (GP-2) that had been found to be an exceptionally rich source of branched oligosaccharides of *N*-acetyllactosamine (blood group I-type antigen) on O-linked oligosaccharides containg a terminal Neu5Gcα2-3Gal linkage which had little binding for human influenza A viruses. In contrast to their binding to GP-2, both hPIV-1 and hPIV-3 showed little binding to equine α2-macroglobulin containing Neu5Acα2-6Gal linkage and Neu4,5Ac2 α2-6Gal linkage on N- linked oligosaccharides which had high affinity for human influenza A viruses. Further analysis using GP-2 modified by enzymatic sialylation revealed that the GP-2 modified with a terminal Neu5Acα2-3Gal linkage had a higher binding activity for the viruses rather than that of intact GP-2. The GP-2 modified with a terminal Neu5Acα2-6Gal linkage also showed binding activity for hPIV-3, but not hPIV-1. To elucidate the molecular mechanism of receptor specificity of human respiroviruses, we characterized a series of human respirovirus HN glycoproteins whose amino acid residues were mutated. We will describe the receptor specificity of the HN glycoproteins. Our results indicate that hPIV-1 and hPIV-3 preferentially recognize oligosaccharides containing branched *N*-acetyllactosaminoglycans with terminal NeuAcα2-3Gal as receptors and that hPIV-3 also recognizes NeuAcα2-6Gal-containing receptors.

(369) **The Glycosylation of Alpha-1-Acid Glycoprotein in Feline Infectious Peritonitis**

Kerry Cunningham[1], P.David Eckersall[1], Diane.D Addie[1] and Kevin.D Smith[2]
[1] Institute of Comparitive Medicine, Faculty of Veterinary Medicine, University of Glasgow, Glasgow, Scotland, [2] Department of Bioscience, University of Strathclyde, Glasgow, Scotland.

Background: The acute phase response is a pathophysiological host defence system against trauma, inflammation and infection, normally stimulated by pro-inflammatory cytokines, which acts to limit damage and assist in the restoration of healthy tissue following elimination of pathological lesions. It also leads to the increased synthesis and release of a number of plasma 'acute phase proteins' (APP) that function to restore the homeostatic balance. In humans, alpha-1-acid glycoprotein (AGP) is a positive acute phase protein that increases in concentration in the plasma 2 to 5 fold in certain pathophysiological states. It is extensively glycosylated (45%) with five asparagine-linked complex oligosaccharide chains. In normal serum, AGP does not exist in a single form but as a heterogeneous population of glycoforms. Heterogeneity arises through subtle structural differences in monosaccharide sequence and linkages, degree of branching and extent of sialylation. The existence of structurally distinct glycoforms implies a functional diversity since the properties of a glycoprotein are influenced by the structures of its oligosaccharide chains. During several physiological and pathological conditions, not only is the total concentration of AGP altered but the relative proportions of the normal AGP glycoforms have been found to change and abnormal glycoforms are expressed i.e. the oligosaccharide "fingerprint" of AGP is altered. The plasma concentration of AGP is known to be raised in Feline Infectious Peritonitis (FIP) but there is relatively little information on the diagnostic significance of AGP glycosylation in this disease or species. However a recent publication has reported that AGP is desialylated in FIP compared to non-diseased cats.
Methods: The concentration of AGP was measured in peritoneal fluid and plasma submitted to the feline virus diagnostic laboratory of Glasgow Veterinary School using radial immunodiffusion. Thereafter AGP was purified from the remnant of each sample after all diagnostic tests were complete using a method which ensured no desialylation of the oligosaccharide chains or denaturation of the protein structure occurred. The samples were initially precipitated with 30% PEG 8000, followed by affinity, anion and cation exchange chromatography and then desalted. After acid hydrolysis, the monosaccharide composition of each individual AGP sample using High pH Anion-Exchange Chromatography with pulsed amperometric detection was determined in order to highlight differences in glycosylation in comparison to feline and human AGP from normal plasma.
Results: Initial analysis of feline AGP revealed that there is a noticeable difference between healthy and diseased cats in terms of glycosylation. Fucose, Mannose, Galactose and Glucosamine are commonly found on human AGP and feline AGP from FIP infected cats however little or no fucose was found on normal feline AGP.

Conclusion: The glycosylation pattern of feline AGP has novel features and could be diagnostic for the appearance of FIP, and may open up possibilities for treatment.

(370) **Phosphorylcholine-Carrying α-Glycoglycerolipids From *Mycoplasma Fermentans*: Investigation of Stereochemical and Biological Relationships**

Takanori Nakamura[1], Yuko Shingu[1], Kazuhiro Matsuda[2], Götz Milkereit[3], Sven Gerber[3], Volkmar Vill[3], Yoshihiro Nishida[1] and Kazukiyo Kobayashi[1]

[1] Department of Molecular Design and Engineering, Graduate School of Engineering, Nagoya University, Furo-cho, Nagoya 464-8604, JAPAN, [2] National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, JAPAN, [3] Institute of Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, 20145 Hamburg, Germany.

Mycoplasma fermentans is a pathogenic microbe associating with autoimmune diseases, allergy, and cancer. Recently, Matsuda reported the isolation of Glyco-Glycero-Phospho-Lipids (GGPLs) from *M. fermentans* and then important biological activities[1)]. For example, GGPLs are main membrane lipids of *M. fermentans* and play as a specific immunodeterminant of *M. fermentans.* GGPLs are key compounds to unfold the pathogenicity of *M. fermentans*. Our first purpose of this study was to establish the stereoselective synthetic pathway to α-glycosylglycerides. We established a new one-pot α-glycosylation methodology using CBr_4 + PPh_3 (Appel reagents) in DMF solvent[2)]. Using our α-glycosylation, we synthesized various GGPL derivatives including four stereoisomers of GGPL-I, having glucose or galactose in combination with *sn*-1,2 or *sn*-2,3 glycerides.

Our second purpose is to find the correlation between the structures and pathogenic actions. We examined physicochemical properties of four stereoisomers of GGPL-I. Liquid crystal properties were investigated by polarizing microscopy. All four stereoisomers have three phases: smectic A, cubic, and columnar phases. It has been reported that only few compounds have all three phases. Further, the formation of cubic phase is thought to play an important role in transporting and fusion processes of cell membranes. Among the four stereoisomers, the glucose type isomer made more stable layer than the galactose type isomer, and the *sn*-2,3 glyceride isomer made more stable layer than the *sn*-1,2 glyceride isomer. Monolayer properties were investigated by both pressure-area isotherm and surface morphology. In the surface pressure-area isotherms, the four stereoisomers were spread at the air/water interface to form uniform liquid-expanded monolayers. Especially, the glucose type isomer formed less flexible layer than the galactose type, and the *sn*-1,2 glyceride also formed less flexible membrane than the *sn*-2,3 glyceride.

We also examined the biological activities. Inhibition assay of interleukin-10 (IL-10) was investigated. IL-10 is an inhibitory cytokine and is suspected to play important roles in autoimmune diseases including rheumatoid arthritis. It has been revealed that the phosphorylcholine group plays some important roles in inhibition of IL-10. GGPLs also expressed the inhibition activities. The GGPL-I isomer having galactose and *sn*-1,2 glyceride showed the strongest inhibition activity. This activities may be correlative with the physicochemical properties. Now, we believe that the physicochemical character of GGPL-membrane of *M. fermentans* greatly affects the biological activity and pathogenicity.

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(371) **Identification of an Endothelial Cell Surface Receptor Involved in Sialyl-Lewisx-Dependent Cancer Metastasis to the Lung.**

Michiko N. Fukuda, Hiroto Kawashima and Minoru Fukuda

Glycobiology Program, Cancer Research Center, The Burnham Institute, La Jolla, California, USA..

Apical surfaces of epithelial cells are covered by a variety of carbohydrates attached to membrane proteins and lipids. When epithelial cells are transformed, the repertoire of cell surface carbohydrates alters significantly. Many studies suggest that O-glycans with terminal structures such as sialyl Lewis X (sLeX) play an important role in cancer metastasis. Because sLeX is the ligand for selectins and E- and P-selectins are expressed on inflammatory blood vessels, these selectins are thought to be responsible for sLeX-dependent cancer metastasis. To investigate carbohydrate-dependent cancer metastasis in vivo, we developed an sLeX-dependent experimental cancer metastasis model in the mouse. While mouse melanoma B16 cells are negative for sLeX-antigen, they acquire sLeX-antigen after transfection with fucosyltransferase III (FTIII) cDNA. When sLeX positive B16-FTIII-M cells were injected intravenously into mice, B16-FTIII-M cells colonized the lung, whereas sLeX-negative B16 cells did not (1). We also identified several peptides that function as selectin ligands (2). When one such peptide, IELLQAR, was injected intravenously into mice, it bound to lung vasculature and inhibited lung colonization of B16-FTIII-M cells. However, this peptide inhibited cancer colonization in mutant mice lacking both E- and P-selectins, suggesting the existence of a carbohydrate binding receptor or IELLQAR peptide receptor (IPR) distinct from selectins (3). The 46 kDa IPR was isolated by peptide affinity chromatography from rat lung membranes. Mass spectrometry identified IPR as a pre-mRNA splicing factor (SF). Although SF proteins do not have a signal sequence typical of a membrane protein, a biotinylation reagent injected intravenously into mice labeled SF protein, which was immunoprecipitated with an anti-SF antibody and detected by avidin blot. When mouse endothelial F2 cells were transfected with cDNA encoding SF-Flag protein, the Flag-epitope was detected on the cell surface. Finally, upon intravenous injection into mice, anti-SF antibodies inhibited lung colonization of B16-FTIII-M cells in vivo. Although SF shows no homology with carbohydrate binding proteins, carbohydrate and RNA binding activities may overlap, since some galectins have mRNA splicing activity. (1) Ohyama, C. et al., Embo J, 18: 1516-1525, 1999. (2) Fukuda, M. N. et al., Cancer Res, 60: 450-456, 2000. (3) Zhang, J. et al., Cancer Res, 62: 4194-4198, 2002. Supported by DAMD17-02-1-0311 and CA71932 to MNF.

(372) **Increase of GQ1b Gangliosides in the Adult Mouse Hippocampus Following Kindled-Seizures.**

Keiko Kato[1], Masao Iwamori[2] and Yoshio Hirabayashi[3]

[1] Department of Laboratory Animal Medicine, Division of Veterinary Science, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka, 599-8531, Japan, [2] Department of Biochemistry, Faculty of Science and Technology, Kinki University, 3-4-1, Kowakae, Higashi-osaka, Osaka, 577-8502, Japan, [3] Neuronal Circuit Mechanisms Research Group, Brain Science Institute, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama, 351-0198, Japan.

Epilepsy is known as a *seizure disorder* , which is usually defined as a sudden alteration of behavior due to a temporary change in the electrical functioning of the brain. The seizures in epilepsy may be related to a brain strokes, brain tumors, Alzheimer's disease, or a family tendency, but often the cause is completely unknown. About 1% of the world's population may have epilepsy and about 10% of children and up to 15% of adults with epilepsy have seizures that do not respond to treatment. We have noticed on acquirement of abnormal neural plasticity as an important cause of epileptogenesis. Alternatively, glycosylation is one of the most familiar post-translational modifications providing variation to the fundamental molecules. Thus, we have investigated about correlation between glycosylation and epileptogenesis using amygdaloid kindling model mice. The kindling is a model of human temporal lobe epilepsy, in which excitability in limbic structures is permanently enhanced by repeated stimulation, in which adult mice received a biphasic square wave pulse [495 microA, 60Hz, 200 microS duration, for 2 sec] unilaterally in the basolateral amygdaloid complex once a day. Acquirement of kindled-seizures was monitored with behavioral and electro-encephalographical criteria, in which period of the establishment was for almost 4 weeks, the transitional changes were accounted as 5 stages, and finally mice with seizures were used in the experiments. As structural changes, representative changes were observed in the hippocampus, in which the dendritic growth and dendritic bundle formations were observed in the apical dendrite of the granular cell layer of the dentate gyrus, receiving axons directly from the basolateral amygdaloid complex that receives kindling-stimulation. These confirmed that kindling-epileptogenesis was acquired by abnormal neural plasticity containing behavioral, physiological, and dendritic aberrations. Then we have screened differential sialylations in brain during epileptogenesis. We have found that mRNA and the endo-product alpha 2,3-sialyltransferase (ST3Gal IV) increased gradually in neuronal cells during kindling-epileptogenesis. As ST3Gal IV transfers sialic acid into glycoprotein, it was suggested that sialylation on glycoprotein was up-regulated by ST3Gal IV during epileptogenesis. In the present, we observed differential sialylation levels of glycolipids following kindled-seizures. GQ1b among neutral and acidic lipid fractions in mouse hippocampus up-

regulated significantly following kindled-seizures. These suggest that sialylation on glycoproteins and glycolipids plays roles in epileptogenesis.

(373) **Isolation and Characterization of Glycolipids Recognized With Dengue Virus Type 2 From Human and Mosquito Cells**

Kazuya IPJ Hidari[1,2,3], Chie Aoki[1,2,3], Takashi Ogi[1,2,3], Saki Itonori[4], Futoshi Hasebe[3,5], Koichi Morita[3,5], Mutsumi Sugita[4], Tadanobu Takahashi[1,2,3], Chao-Tan Guo[1,2,3,6], Daisei Miyamoto[1,2,3], Takashi Suzuki[1,2,3] and Yasuo Suzuki[1,2,3]

[1] Department of Biochemistry, University of Shizuoka School of Pharmaceutical Sciences, Japan, [2] COE 21st Program, [3] CREST, JST, [4] Department of Chemistry, Faculty of Liberal Arts & Education, Shiga University, Japan, [5] Department of Virology, Institute of Tropical Medicine, Nagasaki University, Japan, [6] Zhejiang Academy of Med. Sci., China.

Dengue virus, a member of the flaviviruses causes serious diseases in tropical and subtropical regions of the world. This pathogen is transmitted to human by infected mosquitoes. There exist no specific treatments for infection and control of dengue virus so far. Characterization of receptor molecules has not been enough to be elusive. Elucidation of molecular mechanisms on the interaction of viral glycoprotein, E-glycoprotein (EGP) with host receptors would be critical determinants to understand host range, tissue tropism and virulence. In this study, we isolated and characterized carbohydrate molecules recognized with dengue virus (DEN) type II from human and mosquito cells. Using TLC/virus-binding assay, we investigated glycolipids which react with dengue virus type 2 in a human cell line, K562 and a mosquito cell line, C6/36. Reactive glycolipids were purified by ion-exchange column and high performance liquid column chromatography (HPLC). Structures of the binding glycolipids isolated were determined by mass spectrometry and immunochemical methods. The inhibitory effect of carbohydrate molecules structurally-related with the purified glycolipids on the infectivity of DEN to host cells was also tested. Virus-binding glycolipids were highly purified by a combination of DEAE-Sepahdex A-25 column chromatography and HPLC equipped with Aquasil column. Structural analyses showed that carbohydrate structures of glycolipids purified from human and mosquito cells were not identical. However, in both cell lines, glycolipids bound to DEN contained lactosamine unit or sugar chains related with it. Treatment of carbohydrate molecules structurally-related with the purified glycolipids significantly reduced both binding and infectivity of DEN to host cells. These results strongly suggested that the glycolipids identified in human and mosquito cells are involved in the binding of DEN to the host cell surfaces. Carbohydrate derivatives from structurally-related with the defined glycolipids would be anti-dengue agents.

(374) **Analysis of the Effects of Heparin Sulphation Patterns on Palatal Fibroblast Activities**

Xiao Hui Zou[1], Weng Chiong Foong[1], Tong Cao[1], Boon Huat Bay[2], Yi Fa Zhou[3] and George Yip[2]

[1] Faculty of Dentistry, National University Hospital, National University of Singapore, Singapore , [2] Department of Anatomy, National University of Singapore,Singapore, [3] School of Life Sciences, Northeast Normal University, P.R. China.

Aim: Palatal fibroblast proliferation, adhesion and migration are fundamental events in wound healing after surgical correction of cleft palate. Heparin and heparan sulphate proteoglycans have been shown to bind to fibroblast growth factors and other signalling molecules and influence their biological actions. In this study, we examined the effects of different heparin sulphation patterns on the biological activities of palatal fibroblast. Methods: Passage 2 palatal fibroblasts, isolated from adult NZW rabbits, were cultured in (a) Dulbecco°Øs modified Eagle°Øs medium, (b) medium supplemented with chlorate, a competitive inhibitor of glycosaminoglycan sulphation, or (c) chlorate-supplemented medium plus heparin with one or more specific sulphate groups removed. The effect on palatal cell adhesion was measured using an MTS assay 8 hours after cell seeding. Cell proliferation was determined 3 and 7 days after seeding. In addition, the rate of cell migration was examined, using an in vitro wound-healing model, at 4, 6, 12, 18 and 24 hours after wounding. Results: Inhibition of glycosaminoglycan sulphation by chlorate treatment resulted in significant reduction of fibroblast proliferation and adhesion. Wound closure rate was decreased in the chlorate-treated group compared with the control group. These biological effects of chlorate treatment could be partially blocked by supplementation of the chlorate-containing culture medium with heparin, de-2/3-O-sulphated heparin or de-N- and de-2/3-O-sulphated heparin but not by de-6-O-sulphated heparin. The molecular structures of these selectively-desulphated heparin species were confirmed using nuclear magnetic resonance. Conclusions: Our findings show that heparin regulates palatal fibroblast proliferation, adhesion and migration, and that the 6-O-sulphate group on the molecule is essential for the biological effects of heparin.

(375) **Apoptosis of Human Leukemia Cells Induced by β-Xylooligosaccharides from Green Alga**

Kuniko Yamaguchi[1], Kana Sumoto[1], Noriyuki Sueyoshi[1], Yoshiya Izumi[2], Makoto Ito[1] and Takashi Nakamura[1]

[1] Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka, Japan, [2] Advanced Technology Research Laboratory, Oji Paper Co., Ltd., Tokyo, Japan.

β-1,3-Xylooligosaccharides were separated from enzymatically hydrolyzed β-1,3-xylan prepared from the green alga *Caulerpa racemosa* var. *laete-virens*, and their action on cultured mammalian cells was examined. [^{3}H]thymidine incorporation into human myelogenous leukemia cell line HL-60 cells was strongly suppressed when the cells were exposed to β-1,3-xylooligosaccharides in a dose-dependent manner. Intranucleosomal DNA fragmentation and chromatin condensation, which are the typical features of apoptosis, were observed when the cells were cultured with 0.5-1.0 mg/ml of β-1,3-xylooligosaccharides for 24-48 h. Activation of a caspase-3-like enzyme occurred upon incubation with β-1,3-xylooligosaccharides at 37°C for 48 h. The addition of an inhibitor of caspase, carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone, to the HL-60 cell culture completely inhibited the elevation of caspase-3 activity and DNA fragmentation. These results seem to indicate that β-1,3-xylooligosaccharide-induced apoptosis is mediated by protease(s) of the caspase family, and that the degradation of chromosomal DNA is caused by deoxyribonuclease-like endonuclease activated by caspase.

(376) **Core Fucosylation of N-Linked Glycans in Lad Ii/Cdg Iic Fibroblasts**

Floriana Fruscione[1], Katsuhisa Noda[2], Eiji Miyoshi[2], Naoyuki Taniguchi[2], Michela Tonetti[1] and Laura Sturla[1]

[1] Department of Experimental Medicine and Center of Excellence for Biomedical Research, University of Genova, Italy, Viale Benedetto XV,1 16132 Genova., [2] Department of Biochemistry, the 21st Century COE Program, Osaka University, Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan..

LAD II/CDG IIc is a rare autosomal recessive disease characterized by a decreased expression of fucose in glycoconjugates on cells surface that results in leukocyte adhesion deficiency and severe neurological and developmental abnormalities. The molecular basis of LAD II/CDG IIc syndrome has been identified as a defect in GDP-L-fucose transporter of the Golgi system which reduces the availability of the Golgi GDP-L-fucose, a common donor substrate for fucosyltransferases. Analyses of residual fucose content in different classes of glycoproteins revealed that fucose content is severely reduced in N-linked oligosaccharides, while the levels of O-fucosylation are comparable to control cells (1). It is presently unknown if a limited supply of GDP-L-fucose inside the Golgi could differentially affect the activity of the several alpha-1,2, alpha-1,3/4 and alpha-1,6 fucosyltransferases and if specific types of terminal fucose linkages were more affected than others in LAD II fibroblasts. In the present study,we analyzed N-linked glycans from LAD II skin fibroblasts, with the aim to determine the effects of a reduced GDP-L-fucose availability on the amount of alpha-1,6 fucose (core fucose), compared to alpha-1,2 and alpha-1,3/4 linked fucose. It was found that although all types of terminal fucose in N-linked glycans were decreased in LAD II fibroblasts, total core fucosylation was more heavily affected than fucosylation of outer arms. Confirming our previous data (1), a six-fold reduction in the incorporation of total [^{3}H]fucose in N-linked oligosaccharides from LAD II cells compared to controls was observed, while radioactivity associated to core fucose showed a ten-fold decrease. Moreover, glycans from LAD II fibroblasts showed significantly different patterns of core fucosylation. In control cells, core fucosylation was found to be mainly associated to mono- and di-sialylated biantennary glycans, and, to a lower extent, to triantennary species and to high mannose/hybrid type. In LAD II fibroblasts, core fucose was almost absent in biantennary glycans, while the decrease was less pronounced in triantennary (about 5-fold reduction compared to controls) and in the high mannose/ hybrid fraction (about 3-fold reduction). Moreover, core fucose

was also undetectable in mono- and di-charged species, while neutral species were less affected. Importantly, analyses of an alpha1,6-fucosyltransferase, a responsible enzyme for core fucosylation, revealed comparable enzymatic activity and protein expression among control and LAD II cells, indicating that the above decrease for core fucosylation in LAD II cells is not due to low levels of this enzymatic activity. To date, differential effects on fucosylated glycan compositions by reduced Golgi GDP-L-fucose have been related to different K_ms for fucosyltransferases, i.e. O fucosyltransferases have higher affinities for the GDP-L-fucose compared to those adding terminal fucose (1). The present study focused on alpha1,6-fucosyltransferase and further explored that core fucosylation depend not solely on GDP-L-fucose availability, but also on the types of oligosaccharide acceptors. Consequently, the results herein provide some rational evidences that severe reduction in core fucosylation is responsible for some developmental defects in LAD II patients, as have been observed for alpha 1-6 fucosyltransferase knock-out, core fucose deficient mice (N. Taniguchi, personal communications). 1. Sturla, L. et al (2003) J. Biol. Chem., 278, 26727-26733.

(377) **LARGE Restores Functional Glycosylation of α-Dystroglycan in Congenital Muscular Dystrophies with Distinct Glycosylation Defects**

Rita Barresi[1], Daniel E Michele[1], Motoi Kanagawa[1], Hollie A Harper[1], Harry Schachter[2], Ichizo Nishino[3] and Kevin P Campbell[1]

[1] Howard Hughes Medical Institute, Department of Physiology and Biophysics, Department of Neurology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA., [2] Hospital for Sick Children, Department of Biochemistry, University of Toronto, Ontario, Canada, [3] Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Kodaira, Tokyo 187-8502, Japan..

Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease (MEB), and Walker-Warburg syndrome (WWS), are congenital muscular dystrophies with associated similar developmental brain defects. Genes involved in these diseases encode for putative or known glycosyltransferases: mutations in *fukutin* are responsible for FCMD, *POMGnT1* for MEB, and *POMT1* for a percentage of WWS. The proteins mutated in these disorders are hypothesized to function either directly or indirectly in the transfer of sugars to α-dystroglycan (α-DG). In skeletal muscle, dystroglycan has the central function to bridge between cytoskeleton and extracellular matrix forming a robust link that protects the muscle fiber from contraction-induced damage. The receptor activity of α-DG is modulated by the presence of sugar chains, in that abnormal glycosylation of α-DG disrupts the normal ligand binding activity for its extracellular ligands. We investigated changes in the processing and function of α-DG resulting from genetic manipulation of LARGE, the putative glycosyltransferase mutated in myodystrophic Large^{myd} mice and MDC1D patients. We show that overexpression of LARGE induces the synthesis of α-DG species enriched in glycans with high affinity for extracellular ligands. In Large^{myd} mice, LARGE expression ameliorates the dystrophic phenotype. In wild type mice, high levels of LARGE do not cause muscle pathology. Importantly, LARGE circumvents the posttranslational processing defect of α-DG in cells from patients with genetically distinct types of congenital muscular dystrophy, such as FCMD, MEB, and WWS. Glycan-enriched α-DG produced by LARGE gene transfer in patients' cells displays restored receptor functions and acquires the ability to coordinate the organization of laminin on the cell surface. Our findings indicate that modulation of LARGE expression or activity is a viable therapeutic strategy for glycosyltransferase-deficient congenital muscular dystrophies.

(378) **Clinical Applications of Antibody to the Adenocarcinoma Antigen, T-Antigen**

J. Yan[1], J. Heimburg[1], S. Morey[1], O. V. Glinskii[2], V. H. Huxley[2], V. V. Glinskii[3], L. Wild[4], R. Klick[1] and K. Rittenhouse-Olson[1]

[1] Dept. of Biotechnical and Clinical Laboratory Sciences, The University at Buffalo, Buffalo, New York 14214, [2] Dept. of Medical Pharmacology and Physiology, University of Missouri, Columbia, MO 65212, [3] Dept. of Biochemistry, University of Missouri, Columbia, MO 65211, [4] Dept. of Pathology and Anatomical Sciences, The University at Buffalo, Buffalo, New York 14214.

T-Ag is a tumor-associated antigen of carcinomas including breast, colon, bladder and prostate. T-Ag, the disaccharide Gal β 1-3GalNAc α, is proposed to be an important ligand in adhesion and metastasis. Evidence for this role is 1) increased expression of T-Ag in metastatic tumors, and 2) the presence of lectins that bind T-Ag in sites of metastatic tumor growth including the liver, lungs, bone marrow and lymph nodes. T-Ag is a weak immunogen, and many of the antibodies (Abs) which have been produced are IgM, whereas JAA-F11 is an IgG3. Many of the anti-T-Ag Abs which have been tested cause enhanced proliferation of in vitro tumor cell growth, whereas the addition of JAA-F11 to in vitro culture of tumor cells inhibits growth by a modest (20%) but significant extent ($P<0.01$). A mouse breast cancer animal model was selected from several candidates. Indirect cellular EIA was utilized to detect the expression of T-Ag on tumor cell surfaces and immunohistochemical staining was used to determine if T-Ag is expressed on the surface of tissue sections of primary tumor and metastatic lesions. The chosen line, the 4T1 breast cancer cell line, which is T-Ag positive, spontaneously produces metastases than can spread to the lymph nodes, bone, lung, or other organs after subcutaneous injection into the female mouse mammary gland. Surgery was performed when the primary tumors were palpable. Passive immunotherapy with JAA-F11 antibody was compared to treatment with buffer alone in the development of metastasis and survival rates. Although JAA-F11 did not induce significant killing of 4T1 tumor cells in vitro through ADCC or CDC mechanisms and did not inhibit the growth rate of the primary tumor, this antibody extended the survival time of the animals bearing 4T1 cancer ($P=0.0503$) and inhibited the metastases to lungs ($P=0.0155$). In preliminary studies, this Ab to T-Ag, JAA-F11, has also been shown to block the stage of metastasis that involves adhesion of the metastasizing human breast cancer cells to galectin-3 in human vascular endothelium. This indicates that our Ab would have similar tumor binding and anti-metastatic characteristics in humans and have clinical applicability for the treatment of adenocarcinomas.

(379) **Development of a Peptide Mimic to T-Antigen**

J. Heimburg and K. Rittenhouse-Olson

The University at Buffalo, Buffalo, New York 14214.

The Thomsen-Friedenreich Antigen (T-Ag) is a carbohydrate tumor-associated antigen found in high amounts on the surface of several types of tumor cells, including breast, lung, prostate, and ovary. T-Ag expression contributes to the process of cancer cell adhesion and metastasis. Metastatic sites, including lung, liver, bone, and lymph nodes, contain specific lectins that bind T-Ag. Our laboratory has developed a highly-specific, well-characterized IgG monoclonal antibody to T-Ag, called F11. F11 can interfere with T-Ag binding its known ligand, galectin-3, found on vascular endothelium, thus blocking a primary step in tumor metastasis. In addition, the antibody has potential cytotoxic antitumor activity. Thus the development of a vaccine that causes patients to generate antibodies towards T-Ag would have great clinical value. However, carbohydrate antigens generate T-cell independent responses in the body. More effective are T-cell dependent responses, generated by peptides and proteins. Therefore, identifying peptides that can mimic T-Ag is desirable. Other groups have developed antibodies to saccharide antigens in response to peptide mimics, and demonstrated that animals immunized with peptide mimics have a memory response when immunized with the saccharide. In terms of a response to carbohydrate-epitope bearing tumor cells, current research shows that T cells primed by peptide mimics can then react with carbohydrate molecules to produce cellular responses, and MHC molecules on antigen presenting cells normally displaying peptides can also display glycopeptide moieties. This leads us to the hypothesis that vaccinations using a unique peptide mimic of T-Ag conjugated to a carrier protein will be able to generate cellular immune responses to T-Ag epitopes on tumor cells which will be useful clinically in active immunotherapy of cancer. Our laboratory has identified peptide sequences able to mimic T-Ag, as demonstrated first by the ability of F11 to bind the peptide mimics in immunoblotting experiments. The peptides can block F11 binding to T-Ag as seen by inhibition ELISA experiments (up to 50% inhibition). To measure the affinity of F11 for the peptides versus T-Ag, Biacore analysis and affinity ELISA~{!/~}s were performed (F11 affinity for peptide: KD=5.73E-04). The peptides were shown to block rolling and stable adhesion of cancer cells to the vascular endothelium in an in vivo model system (up to 50% inhibition of rolling adhesion and up to 81% inhibition of stable adhesion). This inhibition shows specificity of the peptide-galectin-3 interaction and potential of the peptides to block adhesion, an important step in metastasis. The peptide mimics were conjugated to carrier proteins and used to immunize rabbits and mice. Continuing experiments will analyze the animal sera by ELISA for the production of antibodies reactive to T-Ag, and analyze spleen cells for reactivity to T-Ag by proliferation and cytotoxicity assays. Because T-Ag is cryptic on normal

cell surfaces, there will be minimal cross-reactivity with normal tissue and the response will show specificity for T-Ag-bearing tumor cells. This research has the potential to be used to decrease the tumor burden of cancer patients as well as aid in prevention of newly formed tumors by blocking cancer cell adhesion and metastasis.

(380) **Chromatographic Separation of Oligosaccharide Mixtures Labeled with New Multifunctional Tags**

Andreas H. Franz, Soo Jin Chang and Joanne Hsu
Department of Chemistry, University of the Pacific, 3601 Pacific Avenue, Stockton, CA 95211.

New multifunctional tags for labeling carbohydrates by reductive amination were synthesized. The tags combine UV-activity, bioaffinity, and the possibility for quaternization. The structural identity of the tags was confirmed by NMR spectroscopy, by MALDI-TOF, and by ESI mass spectrometry. Several mixtures of monosaccharides and oligosaccharides labeled with the new multifunctional tag were separated by HPLC on porous graphitized carbon (PGC) and C18 media with acetonitrile/water/TFA gradients. The efficiency of separation was verified by off-line MALDI-TOF mass spectrometry. The linear oligosaccharide maltotriose and the human milk sugars LNFP 2 and LNDFH 2 were labeled as standards with and without deuterium coding. H/D mixtures of known compositions were analyzed for relative abundance by isotope patterns in MALDI-TOF mass spectrometry. Good agreement between predicted and observed values was observed.

(381) **The Elusive Role of Heparan Sulfate in Toxoplasma Gondii Infection**

Joseph R Bishop and Jeffrey D Esko
Cell and Molecular Medicine Glycobiology Research and Training Program, University of California, San Diego, La Jolla, California, 92093-0687.

We have set out to determine the role of heparan sulfate in Toxoplasma gondii infection. Using Chinese hamster ovary cell mutants defective in various stages of heparan sulfate assembly we have shown that heparan sulfate enhances infectivity. Furthermore, infection appears to depend on overall sulfation of the chains. Treating cells with chlorate, an inhibitor of macromolecular sulfation, recapitulated the dependence on heparan sulfate. To examine if heparan sulfate is important for T. gondii infection in other cell types, we created tissue specific knockout mice for N-deacetylase/N-sulfotransferase-1 using the Cre-loxP technology. Mammary epithelia and hepatocytes purified from mice containing the corresponding tissue specific lesions produced heparan sulfate with about 50% less GlcNSO3, similar to CHO mutants lacking this enzyme. In vitro, infection by T. gondii was reduced as well. This effect was not due to altered attachment but rather was due to defective replication after invasion. Surprisingly, when mutant animals were infected with T. gondii, no effect on the rate of infection of deficient tissues was noted as measured with a new Real-time PCR assay. These findings suggest that although heparan sulfate enhances the rate of replication of T. gondii in cells, this effect does not manifest in altered tissue tropism in vivo.

(382) **Production and Characterization of Mice Transgenic for IgM Anti-Ganglioside GD2 Antibody.**

Ikuo Kawashima [1], Yukiko Yoshida[1], Chouji Taya[2], Hiroshi Shitara[2], Hiromichi Yonekawa[2] and Tadashi Tai[1]
[1] Department of Tumor Immunology, Tokyo Metropolitan Organization for Medical Research, The Tokyo Metropolitan Institute of Medical Science, JAPAN, [2] Department of Laboratory Animal Science, Tokyo Metropolitan Organization for Medical Research, The Tokyo Metropolitan Institute of Medical Science, JAPAN.

Gangliosides, sialic acid-containing glycosphingolipids, have been implicated as tumor-associated antigens in various tumors especially in neuroectodermal derived-cancers. Antibodies against gangliosides have been shown to possess strong anti-tumor effects both in vitro and in vivo and to be effective for killing tumors not only in mice , but also in humans. In general, IgG type antibodies are much more effective than IgM antibodies. In fact, IgG antibodies have both antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), whereas IgM antibodies possess only CDC, but not ADCC in vitro. Clinical studies with human antibodies to gangliosides, however, have clearly shown that IgM antibodies are extremely useful for the treatment of melanoma tumors. Moreover, the titers of IgM antibodies to gangliosides, especially those to GM2, parallel the survival rate of patients with melanoma, suggesting that IgM antibodies, as well as IgG antibodies, also have strong anti-tumors in vivo. However, the mechanisms by which IgM antibodies to gangliosides induce strong tumor suppression in vivo have not been elucidated yet. To investigate this point, we produced and characterized C57BL/6 mice transgenic for IgM antibody to ganglioside GD2. The transgenic (TG) mice showed high IgM, but not IgG antibody titers against GD2 in their sera. No significant clinical symptoms were observed. When EL4 cells, syngeneic T lymphoma that express ganglioside GD2, were injected into TG mice, their prolonged survival was observed. CDC of EL4 cells was mediated with TG mice sera. Neither ADCC with their sera nor cytotoxic T lymphocyte activity to EL4 cells was shown in TG mice. Spleen lymphocytes from TG mice had increased numbers of natural killer (NK) cells, but not of T cells, B cells, or macrophages compared with wild-type mice. Depletion of NK cells with anti-asialo GM1 rabbit serum reduced or abrogated the observed anti-tumor effects, suggesting that NK cells play a major role in tumor eradication or suppression. NK cell activity in TG mice was much higher than wild-type mice. Moreover, TG mice showed prolonged survival after injection with syngeneic B16 melanoma cells, which express GM3, but not GD2 or GD3. Taking these results together, our studies demonstrate that the TG mice have significant anti-tumor characteristics, probably due to CDC and NK cell expansion and activation with anti-ganglioside GD2 antibody.

(383) **Effects of Chondroitin Sulfate On Immune Response in Mice**

Shinobu SAKAI[1], Hiroshi AKIYAMA[2], Tamio MAITANI[2] and Toshihiko TOIDA[1]
[1] Graduate School of Pharmaceutical Sciences, Chiba University, Japan, [2] Division of Food, National Institute of Health Sciences, Japan.

We have reported that chondroitin sulfate (CS) up-regulates the antigen-specific Th1 dominant immune response of murine splenocytes sensitized with ovalbumin (OVA) *in vitro* [Sakai, S. *et al.*, *Immunol. Lett.*, 84(3), 211-6 (2002). Akiyama, H. *et al.*, *Biochem J.*, 382(1), 269-78 (2004)]. In this study, CS was administered orally by ad libitum and/or by gavage (400 mg/kg/day) to BALB/c mice sensitized intraperitoneally with OVA and Alum. The titers of OVA-specific IgE and IgG_1 in mouse serum were determined by ELISA method. The productions of OVA-specific IgE and IgG_1 in mice fed CS (ad libitum) were significantly reduced, and the productions of these antigen specific antibodies in mice fed CS by gavage were likely to be reduced compared with those in control mice. We have also examined the effect of CS intake on the immediate-type hypersensitivity. One hour after antigen (dinitrophenyl-OVA) stimulation, the ears of mice fed CS swelled less than those of control mice. Furthermore, the rise in serum histamine stimulated by antigen challenge in the mice fed CS was likely to be smaller than in controls. We then examined the pattern of cytokine production by splenocytes from mice re-stimulated with OVA *in vitro*. The splenocytes from the mice fed CS (ad libitum) produced less Th2 type cytokines such as interleukin (IL)-5, and IL-10 than those from the control group. In contrast, the splenocytes from the mice fed CS (ad libitum) produced Th1 type cytokines such as interferon-γ, and IL-2 were not different from those from the control group. Furthermore, immunophenotypes analysis in the splenocytes using flow cytometry revealed that proportion of the $CD3\varepsilon^+$ cells in the mice fed CS (ad libitum) was significantly increased than that in the control mice. In the analysis of T cell-subsets, $CD4^+$ cells (helper T cells) and $CD8\alpha^+$ cells (suppressor/cytotoxic T cells) were slightly increased than in controls. Additionally, analysis of the cell surface markers of intraepithelial lymphocytes (IELs) using flow cytometry showed that the populations of $TCR\alpha\beta^+$ cells and $CD4^+$ cells in theÅ@mice fed CS (ad libitum) were higher than that of control mice. Consequently, these findings indicate that the intake of CS may down-regulates the Th2 promoted immune responses, reducing antigen-specific IgE and IgG_1 production and antigen-induced anaphylactic response. The results imply that the intake of CS could prevent the IgE-mediated allergy.

(384) **Implication of Gb3 Expression in vivo in Hemolytic Uremic Syndrome**

Tetsuya Okuda[1], Noriyo Tokuda[1], Shin-ichiro Numata[1], Masafumi Ito[2], Michio Ohta[3], Kumiko Kawamura[3], Joelle Wiels[4], Yuko Fujii[1], Takeshi Urano[1], Orie Tajima[1], Keiko Furukawa[1] and Koichi Furukawa[1]
[1] Department of Biochemistry II, Nagoya University School of Medicine, [2] Department of Pathology, Nagoya University Hospital, [3] Department of Bacteriology, Nagoya University School of Medicine, [4] CNRS UMR 1598, Institut Gustave Roussy.

[Purpose] Verotoxins from *Escherichia coli* O157 strain induce apoptosis in several kinds of cells, and cause hemolytic uremic syndrome (HUS). Globotriaosylceramide (Gb3) is known as a receptor for verotoxins, and the mechanism for the cytotoxicity with verotoxins via the Gb3 was fairly elucidated in in vitro experiments, but pathogenesis of hemolytic uremic syndrome (HUS) is not well understood. In this study, we analyzed the Gb3 expression in vivo and its role in HUS. [Methods] The effects of VT-2 in the 10-15weeks old mice were examined by intravenous administration of VT-2. Expression of Gb3 and Gb3 synthase gene mRNA in murine tissues was analyzed by immunohistochemistry and real time RT-PCR, respectively. [Results and discussion] Mice injected with a lethal dose of VT-2 (0.2μg) died two days after VT-2 injection, showing shivering and convulsion just before the death. Histologic analysis revealed diminution of endothelial cells in glomerulus and swelling of capillary in brain cortex one day after injection, and tissue damage in liver and vacuolation in brain cortex just before lethal. Immunohistologic analysis revealed increased Gb3 levels in the liver after VT-2 administration, and results of real time RT-PCR suggested that Gb3 synthase gene expression levels in all of the lesioned tissues increased after VT-2 injection with slightly different kinetics. Morever, VT-2-injected Gb3 null mice showed neither abnormal signs nor destructive changes in tissues. These results indicate that Gb3 (or globo-series glycolipids) is an only receptor for VT-2, and all clinical features induced by VT-2 including HUS completely depend on the expression of Gb3 and its derivatives in vivo.

(385) **Comparison of the Enzymatic Properties of GnT-IX and GnT-V**

Kei-ichiro Inamori[1], Jianguo Gu[1], Eiji Miyoshi[1], Ichiro Matsuo[2], Yukishige Ito[2], Koichi Honke[3] and Naoyuki Taniguchi[1]

[1] Department of Biochemistry, the 21st Century COE Program, Osaka University Medical School, [2] The Institute of Physical and Chemical Research, RIKEN, [3] Department of Molecular Genetics, Kochi Medical School.

We have cloned a new brain specific β1,6-*N*-acetylglucosaminyltransferase (β1,6GnT) cDNA by a data base search using the sequence of human GnT-V as a query (1). The amino acid sequence of the GnT-V homolog (designated as GnT-IX) was 42% identical to that of GnT-V. The GnT-IX mRNA is expressed only in brain and testis, while the GnT-V mRNA is expressed ubiquitously. GnT-V catalyzes the transfer of GlcNAc from UDP-GlcNAc to the core α1,6-mannose arm in *N*-glycan via a β1,6-linkage. On the other hand, GnT-IX catalyzes the transfer of GlcNAc to both the α1,3- and α1,6-linked mannose arms via β1,6-linkages, forming a unique structure that has not been reported to date. Furthermore, GnT-IX also acts on the same sequence of the GlcNAcβ1,2-Manα in *O*-mannosyl glycan (2), which is one of major *O*-glycans in the brain. Since brain *O*-mannosyl glycan contains GlcNAcβ1,2-Man and GlcNAcβ1,2-(GlcNAcβ1,6-)Man structures, GnT-IX may be responsible for the formation of the 2,6-branched structure in the brain *O*-mannosyl glycan. To understand the physiological functions of GnT-IX and GnT-V, we tried to compare the enzymatic properties of these two enzymes. The activity of GnT-IX was not dependent on metal ions such as Mg^{2+}, Ca^{2+}, and Mn^{2+} and EDTA had no effect on the activity as for GnT-V. The optimal pH of the activity of GnT-IX was between 7.5 and 8.5 in MOPS buffer, whereas that of GnT-V was around pH 6.5. The apparent *K*m value for UDP-GlcNAc of GnT-IX was 0.68 mM when a fluorescence-labeled acceptor substrate GnM-S-PAES was used, while the value of GnT-V was 4 mM in the presence of GnGn-bi-PA as acceptor (3). Since these values were relatively higher than those of other GnTs, the difference of the *K*m value for the donor substrate of these two enzymes may be an important factor for biosynthesis of the glycan structures. Taken together, these results strongly suggest that GnT-IX may have a unique function, compared with GnT-V.

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(386) **Novel Glyco Mimic Peptide for Neutralization of Shiga Toxins**

Akio Sakaki, Yoshiko Miura, Masamichi Kamihira, Yuuki Sasao, Shinji Iijima and Kazukiyo Kobayashi

h036612m@mbox.nagoya-u.ac.jp.

Carbohydrate-protein interaction plays an important role in infection of pathogens such as viruses and toxins. Bioactive oligosaccharides have attracted pharmaceutical interest, but their carbohydrate-protein interaction is weak. The amplification of the interaction was studied by multivalent substances such as glycoconjugate polymers, glycopeptides, and liposomes. The interaction was also amplified with carbohyrate mimic compounds such as transition-state analogues and peptides. Especially, the carbohydrate mimic peptides have been paid much attention, because carbohydrate mimic peptides can be cloned within a short time by phage-display peptide library. Here, carbohydrate mimic peptides as an inhibitor against Shiga toxins (Stxs: Stx-1 and Stx-2) were prepared by phage display library. Stxs produced by E. coli O-157:H7 cause serious clinical complications. Gb3Cer (α-D-Gal (1-4) β-D-Gal (1-4) β-D-GlcCer) is a ligand of Stxs and mediates internalization of Stxs into host cells. We have reported the preparation of polymeric substance carrying multivalent Gb3 as a neutralization reagent. In this investigation, we have prepared Gb3 mimic peptides as a novel inhibitor of Stxs using the strong interaction between carbohydrates and peptides. Gb3 mimic peptides were selected by biopanning to anti-Gb3 antibody. The Fab region of the antibody was subjected to selection, and the sequence was examined by phage display subtraction. The biological ability of the selected phage clone was analyzed by ELISA. The selected phage having a peptide sequence (WHWTWLSEY) showed strong affinity to anti-Gb3 antibody, and the affinity was significantly decreased in the presence of Gb3Cer that is the functional resemblance to the phage clone. The phage also showed the strong affinity to Stxs. The Gb3 mimic peptide (WHWTWLSEY) was synthesized, and binding of the peptide to Stxs was analyzed by SPR. The binding affinity of the peptide was larger than that of Gb3Cer. The peptide indicated strong affinity not only to Stx-1 but also to Stx-2, which was distinctive of Gb3 mimic peptide. The peptide completely neutralized Stxs in vitro using Hela cell. We have succeeded in fabrication of Gb3 mimic peptide that is superior to Gb3Cer in Stxs neutralization.

(387) **in vivo Cleavage of α2,6-sialyltransferase by Alzheimer's BACE1**

Shinobu Kitazume[1], Kazuhiro Nakagawa[1], Ritsuko Oka[1], Yuriko Tachida[1], Kazuko Ogawa[1], Yi Luo[2], Martin Citron[2], Hiroshi Shirata[3], Choji Taya[3], Hiromichi Yonekawa[3], James C. Paulson[4], Eiji Miyoshi[5], Naoyuki Taniguchi[5] and Yasuhiro Hashimoto[1]

[1] Glyco-chain Functions Laboratory, The Institute of Physical and Chemical Research, RIKEN, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan, [2] Amgen, One Amgen Center Drive, MS29-2-B, Thousand Oaks, CA 91320, USA, [3] Department of Laboratory Animal Science, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan, [4] The Scripps Research Institute, Department of Molecular Biology, MEM-L71, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA, [5] Department of Molecular Biochemistry & Clinical Investigation, Osaka University Graduate School of Medicine, Suita-shi, Osaka 565-871, Japan.

A characteristic feature of Alzheimer's disease (AD) is deposition of amyloid b-peptide (Ab) in the brain, which is implicated in the pathogenesis of AD. Ab, a 39- to 43-residue peptide, is generated from the amyloid precursor protein (APP) by the action of β-and gamma-secretases. BACE1 (β-site APP-cleaving enzyme 1), a pepsin-like membrane-bound aspartic protease, was recently identified as β-secretase. We previously reported that BACE1 cleaved rat a2,6-sialyltransferase (ST6Gal I) that was overexpressed in COS cells and that the N-terminus of ST6Gal I secreted from the cells (E41 form) was Glu41(1, 2). Here we report that BACE1 gene-knockout mice have one third as much plasma ST6Gal I as control mice, indicating that BACE1 is a major protease that is responsible for cleaving ST6Gal I in vivo. We also found that BACE1-transgenic mice have increased level of ST6Gal I in plasma. Secretion of ST6Gal I from the liver into the plasma is known to be up-regulated during the acute-phase response. To investigate the role of BACE1 in ST6Gal I secretion in vivo, we analyzed the levels of BACE1 mRNA in the liver, as well as the plasma levels of ST6Gal I, in a hepatopathological model, i.e., Long-Evans Cinnamon (LEC) rats. The LEC rat, a model of Wilson disease, has a deletion in the gene for the copper-transporting ATPase gene (ATP7B)(3). Golgi-localized ATP7B is involved in copper secretion into the plasma, which is coupled with ceruloplasmin synthesis and biliary copper excretion. Like patients with Wilson disease, LEC rats suffer from toxic accumulation of copper in the liver and eventually develop hepatitis and then hepatocellullar carcinoma. LEC rats exhibited simultaneous increases in BACE1 mRNA in the liver and in the E41 form of the ST6Gal I protein, the BACE1 product, in plasma as early as 6 weeks of age, again suggesting that BACE1 cleaves ST6Gal I in vivo and controls the secretion of the E41 form. Reference: 1. Kitazume et al. (2001) Proc Natl Acad Sci U S A 98, 13554-13559 2. Kitazume, et al. (2003) J Biol Chem 278, 14865-148716. 3. Mori et al. (1991) The LEC rat, a new model for hepatitis and liver cancer, Springer-Verlag, Tokyo

(388) **Transcriptional Regulation of the Human β-1,4-Galactosyltransferase V Gene By Transcription Factor Sp1 in Cancer Cells**

Takeshi Sato and Kiyoshi Furukawa
Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173-0015, Japan.

β-1,4-Galactosyltransferase (β-1,4-GalT) V is a constitutively expressed enzyme that can effectively galactosylate the GlcNAcβ1-6Man group of the highly branched N-linked oligosaccharides that are characteristic of tumor cells[1]. Upon malignant transformation of cells, the expression of the β-1,4-GalT V gene increases in accordance with the increase in the amounts of highly branched N-linked oligosaccharides. In the present study, we showed the biological importance of the galactosylation of highly branched N-linked oligosaccharides by the β-1,4-GalT V, and then isolated the promoter region of the human β-1,4-GalT V gene and examined the cis-elements and trans-acting factors that regulate the β-1,4-GalT V gene expression. Lectin blot analysis using RCA-I and L-PHA showed that the galactosylation of highly branched N-linked oligosaccharides is inhibited significantly in SH-SY5Y human neuroblastoma cells by the transfection of the antisense β-1,4-GalT V cDNA, indicating that the β-1,4-GalT V is biologically important for the functions of highly branched N-linked oligosaccharides. We cloned the 2.3-kb 5'-flanking region of the human β-1,4-GalT V gene from a genomic library and found that the GC-rich promoter lacks canonical TATA and CCAAT boxes. To analyze the activity of the promoter in the β-1,4-GalT V gene, we prepared several reporter plasmids consisting of a firefly luciferase gene and the 5'-flanking region of the β-1,4-GalT V gene differed in length. Luciferase assay showed that the β-1,4-GalT V gene promoter is activated mostly in SH-SY5Y cells among cancer cell lines examined, and that the region -116/-18 relative to the transcription start site has promoter activity. The region was found to contain several putative binding sites for transcription factors, including AP2, AP4, N-Myc, Sp1 and USF. Electrophoretic mobility shift assay showed that Sp1 binds to nucleotide positions -81/-69 of the promoter region. Mutations induced in the Sp1-binding site showed that the promoter activity of the β-1,4-GalT V gene is impaired completely in cancer cells. In contrast, the promoter activity increased significantly by transfection of the Sp1 cDNA into A549 human lung carcinoma cells. Furthermore, mithramycin A, which inhibits the binding of Sp1 to its binding site, reduced the promoter activation and expression of the β-1,4-GalT V gene in A549 cells. These results indicate that the expression of the β-1,4-GalT V gene is regulated by Sp1 and that Sp1 plays an essential role in the promoter activation of the β-1,4-GalT V gene in cancer cells. [1] Sato, T., et al. (1998) Proc. Natl. Acad. Sci. USA, 95, 472-477.

(389) **Inhibition of É¿-sheet Aggregation by 6-sulfo-É¿-D-glucosaminides**

Mihoko Koike, Yoshiko Miura, Kikuko Yasuda, Yoshihiro Nishida and Kazukiyo Kobayashi
Department of Molecular Design and Engineering, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Aichi, Japan

A growing number of proteins are being identified that are biologically active though intrinsically disordered, in sharp contrast with the classic notion that proteins require a well-defined globular structure in order to be functional. At the same time recent works showed that aggregation or amyloidosis are initiated in amino acid sequences that have specific physico-chemical properties in terms of secondary structure propensities, hydrophobicity and charges. In intrinsically disordered proteins (IDPs), such sequences would be almost exclusively solvent-exposed and therefore cause serious solubility problems. Further, some IDPs such as the human prion protein, synuclein and Tau protein are related to major protein conformational diseases. Recently, it is clearly shown that structural change of proteins into amyloids cause serious clinical problems such as Alzheimer's, prion and Parkinson's diseases. These Confomational Diseases are based on the conformation change of nerve proteins into É¿-sheet aggregation. It has been pointed out that biological active saccharides have important effects on the conformation transition of these proteins. It is important to investigate the roles of these saccharides in those diseases, which, we believe, leads to the development of novel biological active compounds for medicinal applications. Bioactive saccharides of proteoglycans and gangliosides such as GM1 have been reported to accelerate the amyloid aggregation. Those reports indicated the strong interaction with the proteins or peptides, which motivated us to investigate the interaction with a model saccharide. We have reported that synthesis and biological activity of 6-Sulfo-GlcNAc which behaves mimics of silalic acids and proteoglycans. Here, we investigate the interactions of pNP 6-Sulfo GlcNAc (p-nitrophenyl N-acetyl-6-sulfo-É¿-D-glucosaminides) with representative peptides of Amyloid É¿-peptide (1-42) and prion protein (106-126) for the medicinal applications. First, we investigated the aggregation of AÉ¿peptide with addition of heparin and pNP 6-sulfo-GlcNAc, respectively. The peptide aggregation was estimated by fluorescence of thioflavin T, and the conformation was evaluated by CD spectra. Though heparin induced the É¿-sheet conformation and amyloid formation, pNP 6-Sulfo GlcNAc inhibited them. Other saccharides including glucose and pNP-GlcNAc showed none of such phenomena. Therefore, it was suggested that the sulfate group of pNP 6-sulfo-GlcNAc interacted with AÉ¿ peptide and functioned as a É¿-sheet breaker. The transmission electron micrograph also indicates the inhibition of AÉ¿ aggregation by pNP-6-sulfo GlcNAc. Then, the É¿-sheet breaking effect was also examined with prion peptide. We found that pNP 6-sulfo-GlcNAc sufficiently controlled the aggregation of the peptide by the addition of pNP 6-sulfo-GlcNAc. These results indicated that pNP 6-sulfo GlcNAc works as a É¿-sheet breaker of AÉ¿ and prion protein. Further experiments in vivo and in vitro are under way.

(390) **Sialylation Enhances the Secretion of Neurotoxic Amyloid-Beta Peptide: Regulatory Role of Alzheimer's Beta-Secretase for APP Sialylation**

Kazuhiro Nakagawa[1], Shinobu Kitazume[1], Kei Maruyama[2], Takaomi C. Saido[3] and Yasuhiro Hashimoto[1]
[1] Glyco-chain Functions Laboratory, Supra-biomolecular System Group, Frontier Research System, The Insutitute of Physical and Chemical Reseach (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama, Japan, [2] Department of Pharmacology, Saitama Medical School, Moroyama, Saitama, Japan, [3] Proteolytic Neuroscience Laboratory, Brain Science Institute, The Insutitute of Physical and Chemical Reseach (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama, Japan.

A neuropathological hallmark of Alzheimer disease (AD) is the formation of amyloid plaques in the brain. The amyloid plaque is composed of a neurotoxic peptide, Abeta, which is produced from amyloid precursor protein (APP) by protelyitc cleavage. APP is first cleaved at its beta-site by a membrane-anchored aspartyl protease, BACE1. The beta-site cleavage generates a soluble N-terminal fragment (sAPPbeta) and a membrane-bound C-terminal stub (CTFbeta). The CTFbeta is subsequently cleaved by another protease, gamma-secretase, yielding Abeta and CTFgamma. Thus BACE1 is implicated for triggering the pathogenesis of the disease. We previously demonstrated that BACE1 cleaved alpha2,6-sialyltransferase (ST6Gal-I) and down-regulated the transferase activity (1, 2). In the present study, we have investigated the effect of APP sialylation on its metabolism and regulatory role of BACE1-dependent cleavage of ST6Gal-I. Overexpression of the sialyltransferase in Neuro2a cells accelerated the processing of endogenous APP, but not other proteins, and enhanced the secretion of its metabolites such as sAPPbeta and Abeta. Upon overexpression of sialyltransferase, APP processing was similarly accelerated in CHO cells but not in sialylation-deficient mutant CHO (Lec-2) cells, indicating that the acceleration of APP metabolism or Abeta secretion requires sialylation of cellular protein(s). Furthermore, the overexpression of sialyltransferase did not affect metabolism of a mutant APP that lacked N-glycosylation sites (APP-Asn467,496Ala), suggesting that sialylation of N-glycans on APP itself is essential for the acceleration. Because BACE1 is a protease that cleaves APP, overexpression of BACE1 enhances the secretion of sAPPbeta and Abeta. Å@We speculated that the overexpression of BACE1 affects APP metabolism also by downregulating sialyltransferases and the effect is cancelled for N-glycosylation-deficient APP. Indeed, co-transfection of BACE1 with APP_{NL}, Swedish-type APP, enhanced secretion of sAPPbeta up to 6-fold higher than the control (overexpression of APP_{NL} alone), whereas that of BACE1 with N-glycosylation-deficient APP_{NL} (APP_{NL}-Asn467,496Ala) further enhanced the secretion to 9-fold higher than the control (APP_{NL}-Asn467,496Ala alone). Thus the lack of N-glycans enhanced BACE1-induced sAPPbeta secretion, suggesting that BACE1 regulates APP metabolism not only by cleaving the molecule but also by downregulating sialylation *via* sialyltransferase cleavage. In the autopsy brain suffered from sporadic AD, BACE1 activity is 2~3-fold higher than those of controls, which appears to be primary cause of the disease. In spite of the enhanced activity of BACE1, the pathogenic processes of sporadic AD develop very slowly, taking 10~20 years for manifesting the symptoms. Downregulation of APP sialylation by

the enhanced activity of BACE1 may partly account for the prolonged development of the disease and would be a potential therapeutic target in the future. (1) Kitazume et al.: *Proc. Natl. Acad. Sci. USA*, 98: 13554-13559 (2001) (2) Kitazume et al.: *J. Biol. Chem.*, 278: 14865-14871 (2003)

(391) **Studies on Chemical Properties of Cyclic Sialic Acid Using their Synthetic *S*- and *O*-Glycosides as Model Compounds**

Syoko iwata[1], Chihiro Sato[2,3], Hiromune Ando[5], Makoto Kiso[6], Reiji Kannagi[7] and Ken Kitajima[1,2,3,4]

[1] CREST-JST, [2] Grad.Sch.Bioagr.Sci.,Nagoya Univ., [3] Biosci.Biotech.Center,Nagoya Univ., [4] Inst.Adv.Res.,Nagoya Univ., [5] Life Sci.Res.Center, Gifu Univ., [6] Facul.Agricul.,Gifu Univ., [7] Div.Mol.Pathol.,Aiti Cancer Ctr.Res.Inst..

Three forms of sialic acid (Sia) are demonstrated to occur in the sialyl 6-sulfo Le^X structure: N-acetylneuraminic acid (Neu5Ac), neuraminic acid (Neu), and cyclic sialic acid (cNeu) that may be formed through lactamization of Neu. The sialyl 6-sulfo Le^X structures with these three Sia forms exhibit different binding affinities to selectins. Therefore, it is suggested that the selectin-selectin ligand interaction is regulated by the deacylation of Neu5Ac residue and the lactamization of the Neu residue. The cNeu residue has been only detected in sialyl 6-sulfo Le^X using a specific monoclonal antibody, G159. Our hypothesis is that the cNeu residue occurs not only in sialyl 6-sulfo Le^X, but also in many other sialyl glycan chains in glycoconjugates. To demonstrate our hypothesis, it is necessary to develop chemical methods to detect cNeu residues in any sialyl glycans. In this study, we characterized chemical properties of cNeu and Neu residues to develop a detection method of these residues. Using synthetic compounds, Neu5Ac-*S*-phenol, Neu-*S*-phenol, and cNeu-*S*-phenol, the following properties were revealed. (i) Neu-*S*-phenol was not hydrolyzed under acidic conditions where Neu5Ac-*S*-phenol was hydrolyzed. (ii) cNeu-*S*-phenol appeared to be hydrolyzed under the acidic conditions because thio-phenol and its disulfide compound were formed. (iii) The Neu residue was detected as *N*-acylneuraminic acid (Neu5Acyl) after *N*-acylation followed by the acid hydrolysis. (iv) Neu-*S*-phenol, and cNeu-*S*-phenol were methanolyzed to give rise to the same compound, which could be detected by gas chromatography-mass spectrometry (GC/MS). The similar experiments were also carried out using Neua2-6Glc-SE and cNeua2-6Glc-SE as model *O*-glycosides of Neu and cNeu, respectively. These results suggest that the cNeu residue can be chemically detected using methanolysis GC/MS procedures.

(392) **Regulation of Density of O-Glycans on MUC2 Mucin by Polypeptide Galnac-Transferases Expressed in Colon Carcinoma Cells**

Hideyuki Takeuchi[1], Katsuaki Usami[1], Michihiko Waki[1], Hans Wandall[2], Kentaro Kato[1,2], Henrik Clausen[2] and Tatsuro Irimura[1]

[1] Laboratory of Cancer Biology and Molecular Immunology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Japan, [2] Department of Oral Diagnostics, Faculty of Health Sciences, School of Dentistry, University of Copenhagen, Denmark.

[Background and Aim]

Sites and density of O-GalNAc attachment on mucins are thought to be determined by the orchestrated actions of UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferases (pp-GalNAc-Ts). The density of glycans should play a crucial role in the recognition of mucins by carbohydrate recognition molecules. Such interactions lead to inflammation, infection, and cancer metastasis. Present work is designed to elucidate whether six human colon carcinoma cell lines express distinct sets of pp-GalNAc-Ts and which one of these pp-GalNAc-Ts is the most crucial to determine the density of O-glycans on mucins.

[Methods]

Six human colon carcinoma cell lines (DiFi, KM12C, LoVo, LS174T, MOSER, and T84) expressing MUC2 were chosen. They were compared for the levels of mRNA for 8 pp-GalNAc-Ts (pp-GalNAc-T1, 2, 3, 4, 6, 7, 8, and 9) by competitive RT-PCR. Microsome fractions of these cells were used as a source of pp-GalNAc-Ts and incubated with an FITC-labeled 24-mer peptide corresponding to MUC2 (GTQTPTTTPITTTTTVTPTPTPTG) and UDP-GalNAc. The products of cell-free GalNAc incorporation were separated by RP-HPLC and characterized by MALDI-TOF MS.

[Results and Discussion]

The cell lines were found to be classified into two groups based on the elution profiles of the products. Microsome fractions of the first group (DiFi, KM12C, LoVo) produced highly glycosylated peptides (group 1). The other group (LS174T, MOSER, and T84) produced poorly glycosylated peptides (group 2).

Competitive RT-PCR assays using the competitor and the standard cDNA specific for each pp-GalNAc-T gene were performed. Each cell showed the distinct expression profile of pp-GalNAc-Ts. The expression level of pp-GalNAc-T7 in DiFi, KM12C and LoVo cells (group 1) was higher than that in LS174T, MOSER, and T84 cells (group 2).

293T cells, which lack pp-GalNAc-T7 mRNA, were used as a model to study the effect of addition of pp-GalNAc-T7. A microsome fraction of 293T cells produced poorly glycosylated peptides when a 24-mer MUC2 peptide was used, indicating that this cell line belongs to group 2 though they were not colon carcinoma cells. When recombinant pp-GalNAc-T7 was mixed into the microsome fraction of 293T cells, the mixture produced highly glycosylated peptides dependent on the dose of recombinant pp-GalNAc-T7. Recombinant pp-GalNAc-T7 alone produced poorly glycosylated peptides.

These results suggest that pp-GalNAc-T7 is an important isoform in the regulation of density of O-GalNAc attachment on MUC2. Our preliminary studies indicated that the levels of pp-GalNAc-T7 in epithelial cells were modulated during the differentiation and the activation in response to microenvironmental stimuli. Therefore, altered mucin glycosylation by pp-GalNAc-T7 is proposed to be implicated in the epithelial defense.

(393) **Hypoxia and Free Fatty Acids Induce Changes in Proteoglycan Biosynthesis in Macrophages and Endothelial Cells.**

A Ingemansson, V FridÈn, J Moses, G Camejo and G Bondjers

Wallenberg laboratory for Cardiovascular Research, Sahlgrenska Academy at Gothenburg University, Sweden.

Proteoglycans (PGs) play an important role in the retention of low density lipoproteins (LDL) in the arterial wall. The LDL is retained through its affinity to PGs and this process may be the initial phase in the formation of atherosclerotic lesions. An alteration of the PG biosynthesis could produce a more atherogenic environment in the arterial wall. Low oxygen tension which is found in the lesions, might change the PG biosynthesis. Type 2 diabetes and insulin resistance are key systemic risk factors, characterized by high levels of insulin and free fatty acids (FFAs). These factors might also contribute to a changed PG biosynthesis. We have studied the effects of hypoxia and high levels of FFAs on the PG biosynthesis of macrophages and endothelial cells, both key players in the atherosclerotic process. In human primary macrophages, hypoxia causes a down regulation of syndecan-1 mRNA expression but an up regulation of versican mRNA expression, compared cells cultured at 21 % pO2. We observed a decreased production of the syndecan-1 protein under the same conditions. Hypoxia also induced a change in length of secreted glycosaminoglycans from macrophages. The shorter chains could be a result either of the inability of macrophages to produce long chains during hypoxia or a shift of the isoform of versican. In addition, we found that differences between human blood donors seem to modulate the binding of total glycosaminoglycans from macrophages to LDL. An elevated level of linoleic acid up regulates the mRNA expression of CS-6 and -4 sulphotransferases in human endothelial cells. Secreted chondroitin sulphate galactosaminoglycans from linoleic acid treated endothelial cells, display an increased binding to LDL compared to the control. These observations suggest for the first time that oxygen tension is an important factor of the PG biosynthesis in human primary macrophages. The observations seen in endothelial cells could provide an explanation for the relationship between the early phases of atherogenesis and type 2 diabetes.

(394) **Evaluation of the Underlying Defects Causing the Muscle Destruction in Hereditary Inclusion Body Myopathy**

Marjan Huizing[1], Susan Sparks[1], Paul Savelkoul[1], Emily Gottlieb[1], Carla Ciccone[1], Mao-Sen Sun[1], Daniel Darvish[2], Shahrouz Naiem[2], Goran Rakocevic[3], Marinos Dalakas[3], Donna Krasnewich[1] and William Gahl[1]

[1] Medical Genetics Branch, NHGRI, NIH, Bethesda, MD, [2] HIBM Research Group, Encino, CA,

[3] Neuromuscular Diseases section, NINDS, NIH, Bethesda, MD.

Hereditary inclusion body myopathy (HIBM), an adult onset neuromuscular disorder characterized by progressive myopathic weakness and atrophy, is caused by mutations in *GNE*. *GNE* encodes the rate-limiting, bifunctional enzyme of sialic acid synthesis, UDP-GlcNAc 2-epimerase/ManNAc kinase. Using the specific antibodies VIA4 and IIH6, which detect O-mannosylated epitopes of α-Dystroglycan (DG), we demonstrated hypoglycosylation of muscle α-DG in HIBM patients, possibly the result of a deficiency of free sialic acid. Antibodies to the core α-DG protein, β-DG and laminin-α2 showed normal patterns. These findings, indicating that aberrant O-mannosylation of α-DG underlies the muscle pathology of

HIBM, are supported by additional data: 1. We found normal serum transferrin isoforms in HIBM patients, indicating normal N-glycosylation. 2. We showed residual GNE activity in HIBM myoblasts, which may produce enough sialic acid to sialylate other glycoconjugates (N- and O-GalNAc linked) preferentially over the very rare O-mannosylated glycans. 3. HIBM skeletal muscle has been shown to be hyposialylated (Nishino et al). 4. Abnormal O-mannosylation causes other muscular dystrophies. Muscle Eye Brain Disease and Walker Warburg Syndrome are caused by UDPGlcNAc:Man α-Oβ1 GlcNAc transferase and O-mannosyltransferase I enzyme defects, respectively. DG is an integral protein in the Dystrophin-Glycoprotein Complex (DGC), and codes for two proteins, α-DG and β-DG. α-DG is a peripheral membrane glycoprotein that links the membrane spanning β-DG to the extracellular matrix and to the cytoskeleton through non-covalent interactions. Functional domains in the DG gene include transmembrane and dystrophin binding domains in the β-DG region of the protein, and a mucin-like domain, rich in serine and threonine residues for potential O-linked glycosylation, in the α-DG section of the gene. Defects in the DGC cause certain muscular dystrophies, but no human mutations in DG have been reported, despite the identification of many polymorphisms. Severity and age of onset of clinical symptoms vary widely among HIBM patients, even those with the same GNE mutations and within the same family. We studied correlations between DG-polymorphisms and clinical symptoms in 32 HIBM patients, mainly of Iranian-Jewish descent. All patients carried C2725G in the 3'UTR, as well as three unreported amino-acid substituting polymorphisms: T488G (D163E), C741G (P248A), and T2611C (V871A), which are located outside of functional domains or potential glycosylation sites. Two subjects exhibited G1307A (T436T), located within a potential O-gycosylation site in a conserved area of the mucin-like domain. However, there was no correlation between this polymorphism and clinical symptoms; one patient had onset at age 31 and was wheelchair-bound at age 40, while another had onset at age 22 and walks at age 26. More subjects are needed to draw definite conclusions. In addition, polymorphisms in other genes, like laminin-α2, LARGE, and 2,6-sialyltransferase, may influence clinical severity.

(395) **Mapping of Mucin *O*-glycosylation Patterns in Human Colonic Biopsies**

Jessica M HolmÈn[1], Hasse Karlsson[1], Kristina A Thomsson[1], Henrik Sjˆvall[2] and Gunnar C Hansson[1]

[1] Department of Medical Biochemistry, Gˆteborg University, P.O Box 440, SE-405 30 Gothenburg, Sweden, [2] Department of Physiology, Gˆteborg University, P.O Box 432, SE-405 30, Gothenburg, Sweden.

A mucus layer covers the epithelial cells in the gastrointestinal, respiratory and reproductive tracts of our bodies. It functions as an important protective barrier protecting us from microbial infections and mechanical or chemical injuries. The mucus layer is characterized by its gel-like properties which are mainly attributed to the gel-forming mucins. These are high-molecular mass glycoproteins, produced by goblet cells, and the MUC2 mucin comprises the main component of the mucous gel. The *O*-linked oligosaccharides constitute 80 % of the total mucin mass and have been proposed to be important for maintaining the commensal flora and in a defence for invading microbes.

There are indications that a defective mucosal barrier is taking part in the pathogenesis of several intestinal disorders, as the functional bowel disorders/irritable bowel syndrome (IBS) and inflammatory bowel diseases (IBD, like ulcerative colitis). The gel-forming mucins and their carbohydrates might be important for these diseases. To explore this further, a study of mucin content and glycosylation in colonic biopsies from ascending and sigmoid colon of different patients has been initiated, employing a novel sensitive proteomic and glycoproteomic approach [1] allowing the study of individual biopsies taken during routine clinical coloscopy examinations.

Mucins and other cell mucosal surface components are purified from one single colonic biopsy and separated by composite gel electrophoresis (polyacrylamide-agarose). The proteins are further blotted onto a PVDF membrane and stained by Alcian blue, detecting negatively charged glycoproteins. The *O*-linked oligosaccharides are released by reductive β-elimination from the blotted protein bands and analyzed by liquid chromatography-mass spectrometry (LC-MS).

In the patients analyzed so far, individual differences in the *O*-glycosylation of the major gel-forming colonic mucin, MUC2, have been observed. The main oligosaccharide components found, although in varying amounts, are the isomers GalNAcα1-3/GlcNAcβ1-3-(NeuAcα2-6)GalNAcol together with a structure containing the sulphated Lewis x antigen, all previously found on human mucins of normal descending colon [2]. Several other saccharides, mainly based on core 3, are present in minor amounts, also showing individual differences. Some of the minor saccharides have blood group Sd^a/CAD or sialylated T antigens as terminal determinants. An increase in the sialylation grade in the sigmoid colon as compared to the ascending part was also observed.

The described approach will be used for a wider mapping of the glycosylation of the MUC2 mucin in healthy control patients and compared with patients with functional and inflammatory bowel diseases.

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(396) **O-glycosylation of transcription factors in tumor cell variants which differ in their malignant and metastatic capacity.**

Simon Amzalleg, D Fishman, R Glicklis, S Tsory and S Segal

Department of Microbiology and Immunology, BGU Cancer Research center, Ben Gurion University of the Negev, Beer-Sheva 84105 Israel..

Structural post translational modification of proteins is a process which alters protein conformation and is known to play a regulatory role in the determination of protein activity involved in various major cellular events. Post translational modification of a protein by the addition of a single O-linked N-acetylglucoseamine(O-GlcNAc) residue was initially described in 1984 by Carmen-Rosa Torres and Gerald W. Hart. Since this original observation numerous investigations dedicated to explore this subject demonstrated the ubiquitous nature of this modification, characterized its dynamics and the specific enzymatic machinery involved in the attachment or removal of , linked O-GlcNAc to/from the hydroxyl groups of Ser and Thr residues. These studies also revealed that most of the proteins undergoing this modification are intracellular and restricted almost exclusively to the cytoplasm and nucleus. In light of the fact that Ser and Thr residues can practically be the same or adjacent to sites subject to modification by phosphorylation, this modification may play a major role in structural regulation in a reciprocal manner to phosphorylation and thus, could modulate many major biological processes i.e. metabolism, signal transduction, protein-protein interaction, cell motility etc. The restricted compartmentalization and the effects of this modification on various protein complexes and components of the transcription machinery (including transcription factors) and previous results obtained in our laboratory, which demonstrated the existence of profound differences in transcriptional processes in IC9 and IE7 variants of the highly malignant T-10 murine fibro sarcoma were and still serve as guidelines to our present study. In our previous work we demonstrated the existence of differences in O-Glycosylation of major transcription factors complexes such as NFkB and AP1 in the aforementioned clones which express different sets of MHC class I glycoproteins and differ in their malignant and metastatic potential. Our present investigation was dedicated to the exploration of the exact composition and stoichiometric relationship of the different proteins in the protein oligo nucleotide complex and further examination of other proteins of interest that may poses differential O-glycosylation patterns. This investigation is mandatory in order to better understand the origin of the observed differences in tumor progression and signal transduction between these variant tumor cell lines and correlate these differences to their malignant phenotype. Preliminary result of our study using EMSA, super shift assays with appropriate antibodies supported by immunoblotting and precipitation using WGA (wheat Germ Agglutinin- a specific lectin which recognizes N-acetylglucoseamine residues) we have demonstrated the existence of an O-glycosylated form of the P-65 subunit of the NFkB transcription Complex and also were able to demonstrate the existence of a small fraction of an O- glycosylated form of the transcription factor c-fos. Additional results achieved by precipitation experiments using WGA demonstrated the existence of a differential expression of the O-glycosylated form of the major transcription factor ,-Catenin. These findings raise an intriguing possibility that post translational modifications by O-glycosylation could play a determinative regulatory role in addition to phosphorylation and other post translational modifications in the determination of the malignant phenotype of tumor cells.

(397) **An Essential Role for Galectin-12 in Macrophage Activation**

Ri-Yao Yang, Lan Yu, Daniel K. Hsu and Fu-Tong Liu

UC Davis School of Medicine, Research III 3100D, 4645 Second Avenue, Sacramento, CA95817.

Galectin-12 is a two-CRD (carbohydrate-recognition domain) galectin preferentially expressed in leukocytes and adipocytes. The N-terminal CRD is similar to those of other galectins, while the C-terminal one exhibits significant divergence. We previously showed that galectin-12 gene is upregulated when cells are blocked at the G1 or G1/S boundary of the cell cycle, and ectopic expression of this gene causes cell cycle arrest at the G1 phase. Galectin-12 is required for adipocyte differentiation and knockdown of endogenous galectin-12 results in defective adipogenic signaling. Here we show that galectin-12 plays an essential role in macrophage activation. Suppression of endogenous galectin-12 expression in the murine macrophage cell line RAW 264.7 with siRNAs greatly reduced the responses of these cells to LPS and IFNgamma stimulation, as judged by the production of nitric oxide and the inflammatory cytokine TNFalpha. We further determined that the impaired responses were due to defects in the activation of the transcription factors NF-kappaB and STAT-1 in galectin-12 knockdown cells after LPS and IFNgamma stimulation. These results suggest an essential role for galectin-12 in macrophage activation, which is a major event in innate immune response.

(398) **Regulation of Lipooligosaccharide Sialylation in *Haemophilus influenzae***

Simon Allen[1], Laura Greiner[2], Alyssa Morgan[2], Anthony Zaleski[2], Michael A Apicella[2] and Bradford W Gibson[1,3]

[1] Buck Institute for Age Research, Novato, California, [2] Department of Microbiology, University of Iowa, Iowa City, Iowa, [3] Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, California.

Haemophilus influenzae is a Gram-negative commensal organism and opportunistic pathogen present in the nasopharynx of most individuals. It is the causative agent of a variety of infections including otitis media, sinusitis and bronchitis. The outer membrane of *H. influenzae* contains a high level of a lipid A-anchored oligosaccharide, lipoologosaccharide (LOS), which is thought to be a major virulence determinant in the action of the pathogen. A number of the expressed LOS glycoforms terminate in sialic acid (NeuAc) and these sialylated glycoforms are thought to play a role in aiding the bacterium in evading the host immune responces. More recently it has been shown that optimal biofilm formation is dependent upon the ability of the organism to utilize sialic acid. *H. influenzae* is incapable of the *de-novo* synthesis of NeuAc and thus must scavenge this acidic sugar from its environment. The fate of NeuAc once it has entered the cell is one of two possibilities: 1) it is converted to CMP-NeuAc by the CMP-NeuAc synthetase (NeuA/SiaB) and subsequently incorporated into the LOS by sialyltransferase or 2) degraded to N-acetylmannosamine and pyruvate by the N-acetylpyruvate lyase (nanA). It is thought that the dynamic balance between these two pathways is responsible ultimately for the extent to which the LOS and biofilm is sialylated. To study the role of these pathways in regulation of the sialylation of LOS a mutant was created lacking a functional nanA gene. The phenotype of this mutant was investigated in a number of ways including qualitative and quantitative studies of the incorpoation of sialic acid into the LOS using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). The data shows that in the absence of a functional degradation pathway the flux of NeuAc into the LOS sialylation pathway increases leading to the 'hyper-sialylation' of the LOS. This suggests that the regulation of LOS sialylation occurs subsequent to transport due in part to the levels at which the various components of the degradation and biosynthetic pathways are expressed. In addition to the nanA mutant, a second mutant in a homolog of *E. coli* nanR (a suppressor of the nan operon) was also studied for its effect on the expression of sialylated LOS glycoforms. Preliminary data shows that the LOS isolated from this mutant contains lower levels of NeuAc suggesting an increase in activity in the NeuAc degradation pathway drawing intracellular NeuAc away from incorporation into the LOS. This phenotype would correlate with the nanR gene product having a role in suppression of the nan operon.

(399) **A Role for wtCFTR in the Localization of Glycosyltransferases in the Golgi**

Andrew D. Rhim, Lidia Stoykova, David Kim, Mary C. Glick and Thomas F. Scanlin

Cystic Fibrosis Center, Children's Hospital of Philadelphia, Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania..

Cystic fibrosis (CF) is a common, lethal genetic disease in which patients lack functional copies of wtCFTR, an apical chloride channel found in epithelial cells. CF airway cells have been shown to have decreased sialylation and increased fucosylation of membrane glycoconjugates compared to non-CF cells. Correction of CF cells with wtCFTR cDNA leads to a reversal of this altered glycosylation (1). However, the mechanism for this effect has not been elucidated. Here we present data to support our hypothesis that wtCFTR is involved in the steady-state organization of Golgi-resident glycosyltransferases (2). Utilizing sucrose velocity gradients, the subcellular distribution of SiaT, FucT, and GalT activity was described in CF cells (IB3 (DF508/W1282X) and CF/T43 (DF508/DF508) and non-CF cells, BEAS-2B, and C38 cells (IB3 cells transfected with wtCFTR). Two trans Golgi resident non-glycosyltransferase proteins (p230, GS27), localized in these cells as controls, were found in relatively the same fractions of the gradients from all cells, CF or non-CF. However, in all CF cells, SiaT, GalT, and FucT activity were found over a range of gradient fractions that did not necessarily overlap with the location of the trans Golgi markers. In contrast, in non-CF cells, glycosyltransferase activities overlapped with the location of Golgi markers and were significantly different from CF cells. The conserved oligomeric Golgi (COG) complex may be involved in the proper localization of proteins in the Golgi (3). One subunit of the complex was reported to be mutated in a congenital disorder with glycosylation abnormalities (4). The distribution of several COG proteins were found to be significantly different in CF cells when compared to the non CF cells as examined by confocal microscopy. Cog 8, a particularly striking example, was found scattered widely across the cytoplasm in IB3 and CF/T43 cells. However, in the non CF cells, BEAS-2B and C38 cells, Cog 8 was localized to a discrete juxtanuclear compartment corresponding to the Golgi apparatus. These studies provide evidence for the role of CFTR in the organization of the Golgi apparatus, as transfection of CF cells with wtCFTR cDNA led to a correction of glycosyltransferase location. The altered distribution of COG proteins leads to the corollary that wtCFTR regulates glycosyltransferases through the intermediary of the COG complex. Supported by the CF Foundation SCANLI00Z0 (T.F.S.) and NIDDK Medical Student Research Fellowship (A.D.R.) (1) Rhim, A.D. et al., (2000) Glycoconjugate J. 17: 385 (2)Scanlin, T.F. and Glick, M.C. (2000). Glycoconjugate J. 17: 617 (3)Oka, T. et al., (2004) Mol. Biol. Cell 15, 2423 (4)Wu, X, et al., (2004) Nat. Med. 10: 518

(400) **Genome-scale Reconstruction of Human Glycosylation Pathways**

Natalie C. Duarte and Bernhard O. Palsson

University of California, San Diego, 9500 Gilman Dr, La Jolla CA 92093-0412.

High-throughput experimental technologies have generated many large-scale biological data sets. The integration of these various data types is key to fully understanding cellular physiology, especially in the elucidation of disease states. Since most processes require the interaction of components at the cell-scale, studying them requires genome-scale integration of cellular components such as genes, mRNA transcripts, and proteins. Starting with annotated genome sequences and extensive literature searches, our group has manually reconstructed genome-scale stoichiometric models of metabolism and transcriptional regulation for several microorganisms, including the yeast *Saccharomyces cerevisiae* and the bacterium *Escherichia coli*. Our current reconstruction of yeast metabolism is being used as the basis for developing a human metabolic model. In addition, we have also begun to reconstruct major human glycosylation pathways. These reconstructions are unique in that they incorporate several biological data types by directly accounting for the relationships between genes, transcripts, and proteins and by including protein localization information. Also, the molecular formula and charge of each molecule is recorded so that simulations with these models require a cell-wide balance of mass and charge.

In this poster we will describe our current progress in reconstructing N- and O-glycosylation pathways, including the number of genes, proteins, and reactions in the stoichiometric network, maps of the pathways, and any gaps or inconsistencies that we have identified in the knowledge base used for the reconstruction.

(401) **Metabolic Inhibition of Tumor Metastasis In Vivo**

Jillian R. Brown, Mark M. Fuster, Ruixia Li, Charlie Glass and Jeffrey D. Esko

Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, La Jolla, CA 92093 USA.

Sialyl Lewis X (sLe^x) is a cell surface carbohydrate antigen expressed on many carcinomas that facilitates tumor metastasis by binding to the selectin class of cell adhesion receptors located on platelets and endothelia. Patient survival studies after surgical resection of tumors indicate higher mortality for those patients whose tumors express sLe^x. Therefore, the development of a pharmacological approach to inhibit sLe^x on tumor cells could improve patient survival. Towards this goal, we have developed a metabolic inhibitor of sLe^x. *In vitro*, peracetylated GlcNAcβ1-3Galβ-O-naphthalenemethanol acts as a primer of oligosaccharide synthesis, diverting the assembly of mucin type oligosaccharides on endogenous glycoproteins. Priming in mouse LLC Lewis lung carcinoma and B16BL-6 melanoma cells results in inhibition of tumor cell sLe^x expression and a concomitant reduction in adhesion to purified P-selectin. Treated tumor cells in a syngeneic immunocompetent mouse metastasis model have reduced survival, resulting in a reduction in pulmonary metastasis. Analogs have been made that no longer act as primers, but which retain sLe^x inhibitory activity. These metabolic inhibitors represent a novel treatment strategy for reducing tumor metastasis.

(402) **Increased Fucosylation of Serum alpha 1-acid Glycoprotein in Cancer Patients**

Shinji Hashimoto[1], Takayuki Asao[1], Junko Takahashi[2], Yuko Yagihashi[2], Toyo Nishimura[2,3], Abby R. Saniabadi[2], Dennis C.W. Poland[4], Willem van Dijk[5], Hiroshi Kuwano[1], Naohisa Kochibe[6] and Shin Yazawa[2,3,7]

[1] Department of General Surgical Science, Gunma University, Graduate School of Medicine, Maebashi, Japan , [2] Japan Immunoresearch Laboratories, Takasaki, Japan, [3] Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan, [4] Department of Clinical Chemistry, Diaconessenhuis, Leiden, The Netherlands, [5] Department of Molecular Cell Biology and Immunology, VU Medical Center, Amsterdam, The Netherlands, [6] Department of Biology, Faculty of Education, Gunma University, Maebashi, Japan, [7] Cooperative Research Center of Gunma University, Maebashi, Japan.

Alpha 1-acid glycoprotein (AGP) is a major serum glycoprotein with a molecular weight of 41- 43 kDa and its potential physiological significance as an acute phase protein with diverse immunomodulating effects has been investigated. The glycoforms of AGP consisting of five complex di-, tri- and tetra-antennary glycan chains have been shown to change during acute and chronic inflammation, pregnancy and various diseases. Further, the degree of branching and α1,3fucosylation on the tri- and tetra-antennary chains on AGP has also been demonstrated to have various biochemical functions and the role of sialylated Le^X antigen expressed on AGP molecule has been discussed.

In this study, we assayed serum levels of AGP from patients with various cancers by an ELISA and determined the variations in their serum AGP glycoforms during pre- and postoperative periods by crossed affinoimmunoelectrophoresis (CAIE) with Con A and *Aleuria aurantia* lectin (AAL) and anti-AGP antibody[1]. The relative amounts of tri- and tetra-antennary glycan chains were measured by a Con A-anti-AGP gel and those of α1,3fucosylated chains were determined by an AAL-anti-AGP gel. α1,3Fucosyltransferase activity in serum was measured by a RIA using H type 2 conjugated BSA as an acceptor and anti-BSA and anti-Le^Y antibody[2]. A significant difference ($P < 0.001$) of serum AGP levels was observed in preoperative patients compared with those with healthy controls but each level in patients did not reflect their clinical status. Changes in the AGP glycoforms could be easily analyzed with the aid of CAIE and patterns of AGP glycoforms thus determined by CAIE were found not only to be quite different in patients from healthy controls but also to change over a long period after surgery depending on their clinical status. When 45 patients with various clinical status were followed up for a long period after surgery, patients who had no clinical relapse or recurrence so far (well prognosis) (n=34) were found to possess AGP with low branched or fucosylated glycans even though 15 of the 34 patients were classified to have advanced cancer or have metastasis at operation. However, patients who had a relapse and subsequently died (poor prognosis) (n=11) possessed AGP with highly branched and fucosylated glycans for more than 2 months. Di- or trimeric Le^X determinants on branched glycans containing tri- and tetra-antennary sugar chains were suggested to be present in such serum samples. A high correlation ($r = 0.772$, $P < 0.0001$) was observed between the fucosylation of AGP and the serum α1,3fucosyltransferase activities in various cancer patients. The tumor dependent increases in AGP fucosylation therefore must result from changes in the hepatic glycosylation process of AGP.

It was concluded that variations in the relative distribution of AGP glycoforms could be appropriate markers of cancer progression and patients whose AGP glycoforms contained highly fucosylated tri- and tetra-antennary sugar chains for a long period after operation were likely to have poor prognosis.

[1]J. Exp. Med., 177:657-66, 1993. [2]Clin. Chem., 37:2081-86, 1991.

(403) **Possible Role of galectin-9 in Lipopolysaccharide-induced Peritonitis in Mouse**

Hiroko Abe[1], Ryusuke Nakagawa[1], Nozomu Nishi[2], Akira Yamauchi[1] and Mitsuomi Hirashima[3]

[1] Department of Cell Regulation, Faculty of medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa Prefecture, 761-0793 Japan, [2] Department of Endocrinology, Faculty of medicine, Kagawa University, [3] Department of Immunology and Immunopathology, Faculty of medicine, Kagawa University.

Galectins are characterized by selective affinity for β-galactoside and play roles in diverse biological processes, including cell adhesion, proliferation, maturation, and apoptosis. They form an evolutionarily highly conserved family and fourteen members have been identified in mammals so far. These family members can be classified into three subtypes according to their structures. The prototype (galectin-1, -2, -5, -7, -10, -11, -13 and -14) has a homodimeric carbohydrate-recognition domain (CRD), and chimera-type (galectin-3) has a single CRD and an extended N-terminal region. The tandem-repeat type (galectin-4, -6, -8, -9 and -12) has tow CRDs connected by a linker peptide. Recently, it has been shown that galectins may play a key role in immune system and also in inflammatory responses. We have previously shown that galectin-8 (Gal-8) appears to be related to inflammatory response since Gal-8 modulates the neutrophil function, such as superoxide production. Galectin-9 (Gal-9), which has been first identified as a T cell-derived eosinophil chemoattractant, belongs to the tandem-repeat type as Gal-8. Since Gal-9 has not only eosinophil chemoattractant activity but also apoptosis inducing activity against tumor cells and activated T cells, possibility that Gal-9 play a role even in inflammation can not be excluded. In the present studies, we analyzed the effect of Gal-9 on lipopolysaccharide (LPS)-induced inflammation in mouse peritoneal cavity, to clarify the involvement of Gal-9 in the inflammatory responses. When LPS was injected into mouse peritoneal cavity, the levels of T helper type 1 (Th1) cytokines in serum were up-regulated. However, injection of mouse Gal-9 (moGal-9) derived from yeast together with LPS into mouse peritoneal cavity resulted in the suppression of LPS-induced up-regulation of those cytokine, although moGal-9 itself failed to induce notable change in the levels of these cytokines. Furthermore, we found that moGal-9 could modify the population of LPS-induced peritoneal exudates cells (PEC) in the peritoneum. These present results suggest that Gal-9 has certain functions in LPS-induced inflammation.

(404) **Effects of Exogenously Administered Mannose and Deaminoneuraminic Acid (KDN) to Mice or Murine Cultured Cells on the Metabolism of KDN**

Shinji Go[1,2], Chihiro Sato[1,2] and Ken Kitajima[1,2,3]

[1] Grad. Sch. Bioagr. Sci., Nagoya Univ., [2] Biosci. Biotech. Center,Nagoya Univ., [3] Inst. Adv. Res., Nagoya Univ.

Sialic acids (Sia) are a family of nine carbon, 2-keto-3-deoxy sugars found in viruses, bacteria, and higher animals. KDN (deaminoneuraminic acid, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) constitutes a unique group of Sia family because it possesses distinct properties, such as insensitivity to various sialidases, the presence at the terminal position of polysialic acid as a stop signal for the elongation, and the higher levels of expression in certain cancer cells. We previously demonstrated that KDN monosaccharide is synthesized by the following three sequential reactions in rainbow trout testis [Angata et al. (1999) J. Biol. Chem. 274, 22949-22956]: (i) Mannose (Man) + ATP → Man-6-P + ADP; (ii) Man-6-P + phosphoenolpyruvate → KDN-9-P + Pi; (iii) KDN-9-P → KDN + Pi. In mammalian cells, KDN is usually expressed at very low levels, although the expression of KDN is elevated in certain tumor cells. Recently we demonstrated that the level of free KDN in mammalian cells increases when the cells are cultured in mannose-rich media [Angata et al. (1999) Biochem. Biophys. Res. Commun. 261, 326-331]. These results suggest that mammalian cells have a potential to express the high levels of KDN and that some mechanism to suppress the expression of KDN may operate in normal state of the cells. Thus, our objective is to understand the regulation mechanism of KDN expression in mammalian cells. In this study, we studied effects of exogenously administered Man or KDN to mice or mouse cultured cells on the metabolism of KDN in the organs or the cells. 1. We examined the effects of oral ingestion of Man to mice on the level of KDN amount in

their organs. Man were administered to mice by a single oral ingestion or by feeding with water containing 5% Man for two weeks, and the excised organs were analyzed for free and bound forms of KDN by the fluorometric HPLC analyses. In lung, spleen and kidney, the amount of free KDN increased when the mice were fed with water containing Man, while no increase was observed in liver. In all these organs, bound KDN remained unchanged at very low levels. 2. KDN was administered to mice by the oral ingestion, and the uptake of KDN in blood was evaluated by the fluorometric HPLC analyses. The concentration of KDN in blood rapidly (within 30 min) increased after the oral ingestion of KDN. Varying amounts of KDN were also added to culture media of mouse B16 melanoma cells. The intracellular free KDN in B16 cells increased in a dose-dependent manner, and greatly exceeded those levels that were attained when the cells were cultured in Man-rich media. However, bound KDN was not much increased. These results indicate that mouse cells efficiently take up KDN, but do not efficiently utilize it for the synthesis of bound KDN in glycoconjugates. This work has been supported in part by CREST of JST.

(405) **Exploring the Role of the Glycosylation of Mucins in the Defence against Pathogens in the Respiratory and Gastrointestinal Systems**

Niclas G. Karlsson, Benjamin L Schulz, Nicole L. Wilson and Nicolle H. Packer

Proteome Systems Ltd, locked bag 2073, North Ryde Sydney, New South Wales, 1670, Australia.

The highly glycosylated mucin glycoproteins are the main components of the mucus and are present on all mucosal surfaces. The glycosylation on these proteins is believed to be important for the interaction of mucins with invading pathogens. Specific bacterial adhesins have evolved with specificity towards oligosaccharide epitopes present on mucins such as Lewis^b (*H. pylori* BabA adhesin) and sialyl Lewis^x (*P. aeruginosa*, unknown adhesin). A mass spectrometric approach using graphitised carbon LC-MS and LC-MS^2 has been developed with low femtomole sensitivity for studying mucin oligosaccharides released from mucin components isolated by composite gel electrophoresis. Data will be presented that shows that the host response towards a microbial infection may be to alter the glycosylation of mucins and hence the binding of the bacterial adhesins to the mucus layer. We show that some of the altered glycosylation found on the respiratory mucins in the lungs of cystic fibrosis patients is not a primary effect of the defect CFTR ion channel, but rather a secondary effect of the inflammation/infection that these patients are susceptible to, especially by *P. aeruginosa*. The results suggest that oligosaccharide structures containing sialyl Lewis^x is indeed up-regulated in exacerbated cystic fibrosis lungs. Preliminary LC-MS data from a murine model of intestinal infection will also be shown, where mucin glycosylation is altered in respect to increased fucosylation accompanying *Camplyobacter jejuni* infection. This model will allow us to further explore the link between pathogen infection and host glycosylation.

(406) **Subcellular Localization of Fukutin and Fukutin-related Protein in Muscle Cells**

Satoru Noguchi[1], Hiroshi Matsumoto[1,2], Kazuma Sugie[1], Megumu Ogawa[1,3], Kumiko Murayama[1], Yukiko K Hayashi[1] and Ichizo Nishino[1,3]

[1] Department of Neuromuscular Research, National Institute of Neuroscience, NCNP, Tokyo Japan, [2] Department of Pediatrics, National Defense Medical College, Saitama, Japan, [3] Core Research for Evolutional Science and Technology, JST Corporation, Saitama, Japan..

Fukuyama-type congenital muscular dystrophy and congenital muscular dystrophy 1C are congenital muscular dystrophies that commonly display reduced levels of glycosylation of α-dystroglycan in skeletal muscle. The genes responsible for these disorders are fukutin and fukutin-related protein (*FKRP*), respectively. Both gene products are thought to be glycosyltransferases, but their functions have not been established. In this study, we determined their subcellular localizations in cultured skeletal myocytes. FKRP localizes in rough endoplasmic reticulum, while fukutin localizes in the cis-Golgi compartment. FKRP was also localized in rough endoplasmic reticulum in skeletal muscle biopsy sample. Our data suggest that fukutin and FKRP may be involved at different steps in *O*-mannosylglycan synthesis of α-dystroglycan, and FKRP is most likely involved in the initial step in this synthesis.

(407) **Reduction of UDP-GlcNAc 2-epimerase/ManNAc Kinase Activity and Sialylation in Distal Myopathy with Rimmed Vacuoles**

Ichizo Nishino[1,2], Satoru Noguchi[1], Yoko Keira[1,2], Kumiko Murayama[1], Megumu Ogawa[1,2], Masako Fujita[1,2], Genri Kawahara[1,2], Yasushi Oya[3], Yukiko K Hayashi[1] and Ikuya Nonaka[3]

[1] Department of Neuromuscular Research, National Institute of Neuroscience, NCNP, Tokyo, Japan , [2] Core Research for Evolutional Science and Technology, JST Corporation, Saitama, Japan, [3] National Center hospital for Mental, Nervous, and Muscular Disorders, NCNP, Tokyo, Japan.

Distal myopathy with rimmed vacuoles is an autosomal recessive muscle disease with preferential involvement of the tibialis anterior and sparing quadriceps muscles in young adulthood. In Japanese patient with distal myopathy with rimmed vacuoles, we identified pathogenic mutations in the gene encoding the bifunctional enzyme, UDP-GlcNAc 2-epimerase/ManNAc kinase, which catalyzes the initial two steps in the biosynthesis of sialic acid. In this study, we demonstrate the relationship between the genetic mutations and enzymatic activities using an *in vitro* expression assay system. Furthermore, we also show that the levels of sialic acid in muscle and primary cultured cells from patients were reduced to 60-75% of control. The reactivities to lectins were also variable in some myofibers, suggesting that hypo-sialylation and abnormal glycosylation in muscles may contribute to the focal accumulations of autophagic vacuoles, amyloid deposits, or both in patient muscle. Addition of ManNAc and NeuAc to primary cultured cells normalized sialylation levels thus demonstrating the therapeutic potential of these compounds for this disease.

(408) **Dangerous Chicken H5 Influenza Viruses Recognize Sulfated Receptor, Neu5Acα2-3Galβ1-4(6-HSO$_3$)GlcNAc**

Nicolai Bovin[1], Alexandra Gambaryan[2], Alexander Tuzikov[1] and Galina Pazynina[1]

[1] Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry RAS, 117997, ul. Miklukho-Maklaya, 16/10, Moscow, Russia, [2] Chumakov Institute of Poliomyelitis and Viral Encephalitides Moscow, 142782, Russia.

Chicken H5N1 viruses have been isolated from a human in 1997. Eight people with influenza H5N1 infection were hospitalized in Hong Kong and six of these patients died. A number of similar outbreaks was documented in different countries during 2003/2004 season. Normally, avian viruses recognize Sia2-3 receptors and are unable to infect humans. What is the reason of unusual transmission of chicken viruses to humans? What is the nature of their receptor? To characterize the receptor-binding specificity of H5 viruses, we have synthesized a number of oligosaccharides including sulfated ones and used their PAA-conjugates for revealing the receptor specificity. Duck viruses bound with highest affinity to Neu5Acα2-3Galβ1-3GlcNAc (type 1 chain); substitution of a hydroxyl group to sulfate had no effect on binding. By contrast, chicken and human viruses isolated in 1997 in Hong Kong as well as viruses of 2003/2004 season bound sulfated trisaccharide of type 2, Neu5Acα2-3Galβ1-4(6-HSO$_3$)GlcNAc, 6-O-Su-3'SLN, with extraordinary high affinity, 10-100 times stronger then that of the non-sulfated saccharide. Binding of the viruses to tracheal epithelial tissue of green monkey was decreased tenfold after the treatment of the tissue with specific glucosamine-6-sulfatase, suggesting the presence of 6-O-Su-3'SLN in the airway of human epithelium. The presence of 6-O-Su-3'SLN receptor both in chicken and human respiratory tract could explain the direct transmission of the mentioned H5N1 viruses from chicken to humans.

(409) **Down-regulation of É¿1,3-*N*-Acetylglucosaminyltransferase 6 (É¿3Gn-T6; Core 3 synthase) Expression in Gastrointestinal Carcinomas-Overexpression of Core 3 Synthase Suppresses Lung Metastasis**

Toshie Iwai[1,2], Takashi Kudo[3], Akira Togayachi[1], Toru Hiruma[4], Tomoko Okada[5], Toru Kawamoto[6] and Hisashi Narimatsu[1]

[1] Glycogene Function Team, Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), OSL C-2, 1-1-1 Umezono, Tsukuba, Ibaraki, 305-8568, Japan, [2] Laboratory of Glycobiology, The Noguchi Institute, 1-8-1 Kaga, Itabashi, Tokyo, 173-0003, Japan, [3] Department of Anatomy and Embryology, Biomolecular and Integrated Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki, 305-8578, Japan, [4] Research & Development Division, Fujirebio Inc., 51 Komiya-cho, Hachioji, Tokyo 192-0031, Japan, [5] Institute for Biological Resources and Functions, AIST, C-6, 1-1-1 Higashi, Tsukuba, Ibaraki, 305-8566, Japan, [6] Department of Gastrointestinal Surgery, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki, 305-8575, Japan.

The core 3 structure of the *O*-glycan, GlcNAcÉ¿1-3GalNAcÉø1-serine/threonine, an important precursor in the biosynthesis of mucin-type glycoproteins, is synthesized by É¿1,3-*N*-acetylglucosaminyltransferase (É¿3Gn-T; core 3 synthase) on GalNAcÉø1-serine/threonine. Recently, we

cloned and characterized the gene encoding UDP-GlcNAc:GalNAc-peptide É¿1,3-*N*-acetylglucosaminyltransferase 6 (É¿3Gn-T6), and concluded that É¿3Gn-T6 is the core 3 synthase based on its substrate specificity, the linkage structure of the reaction product and the gene expression profile.
The core 3 structure has been reported to be the major form among *O*-glycans in normal colonic tissue, however, its expression is down-regulated in colon cancer tissues. We generated an anti-É¿3Gn-T6 monoclonal antibody and performed immunohistochemical analyses. In normal stomach and colon, É¿3Gn-T6 was strongly expressed in the Golgi region of epithelia. In contrast, its expression was markedly down-regulated in gastric and colorectal carcinomas. Tissue specimens from a familial adenomatous polyposis patient showed a clear correlation between the down-regulation of É¿3Gn-T6 expression and the degree of dysplasia/neoplasia. *In vitro*, the level of *É¿3Gn-T6* transcript was increased according to the differentiation of Caco-2 cells. These results suggested that the expression of É¿3Gn-T6 is closely regulated during differentiation and dedifferentiation. É¿3Gn-T6 would be a useful marker for distinguishing between benign adenomas and premalignant lesions.
To evaluate the relationship between the expression of É¿3Gn-T6 and cancer metastasis, HT1080 FP-10 cells, highly metastatic variant of human fibrosarcoma cells, were transfected stably with the *É¿3Gn-T6* gene. Transfectants showed a decrease of the core 1 structure, GalÉ¿1,3GalNAcÉø1-serine/threonine, probably due to competition between the core 1 synthase and the core 3 synthase. *In vitro* migration activity of the transfectants was markedly lower than that of mock transfectants, and lung metastasis after intravenous injection of the transfectants into nude mice was significantly suppressed. These facts indicated that the core structures of *O*-glycans are profoundly involved in the metastatic capacity of cancer cells.
This work was supported by the New Energy and Industrial Technology Development Organization (NEDO).

(410) **Species-specific Interaction between Human and Murine Lysosomal Beta-Hexosaminidase A Subunits for GM2 Ganglioside Degradation**

Kohji Itoh[1,4], Aya Kuroki[1], Fumiko Matsuzawa[2,4], Sei-ichi Aikawa[2,4], Yasuhiro Ishibashi[1], Daisuke Tsuji[1,4], Hitoshi Sakuraba[3,4] and Hirofumi Doi[2,4]

[1] Department of Medicinal Biotechnology, Institute of Medicinal Resources, Graduate School of Pharmaceutical Sciences, The University of Tokushima, Tokushima 770-8505, Japan, [2] Celestar Lexico-Sciences, Inc., Mihama-ku, Chiba 261-8501, Japan, [3] Department of Clinical Genetics, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, Bunkyo-ku, Tokyo 113-8613, Japan, [4] CREST, JST, 4-1-8 Honcho, Kawaguchi, Saitama, Japan.

ÅyIntroductionÅzTay-Sachs disease and Sandhoff disease are autosomal recessive GM2 gangliosidoses caused by the primary defect of lysosomal β-hexosaminidase (Hex) subunits α and β, encoded by two different genes, *HEXA* (Chr. 15q23-q24) and *HEXB* (Chr. 5q13), respectively, associated with excessive accumulation of GM2 ganglioside (GM2) in the central nervous system and neurological manifestations. There are three Hex isozymes in mammal; HexA (αβ), HexB (ββ) and HexS (αα), but only HexA degrades GM2 ganglioside (GM2) in cooperation with GM2 activator protein. ÅyPurposeÅzTo elucidate the molecular mechanism of interaction between Hex α- and β-subunits and design an artificial HexA molecule with higher function for enzyme replacement therapy of GM2 gangliosidoses, we constructed the human and murine HexA models based on the structural information about the human HexB previously determined. We also examined the effects of normal gene transfer and recombinant enzyme replacement on degradation of GM2 in the fibroblasts with GM2 gangliosidoses. ÅyResultsÅzWe established Chinese hamster ovary (CHO) cells stably overexpressing the human α-, human β-, murine α- and murine β-subunit genes (*HEXA*, *HEXB*, *hexa* and *hexb*), respectively. Each CHO cell line secreted Hex isozymes enzymatically active toward artificial substrates into the culture medium. The conditioned media from the cell lines independently expressing *HEXA* and *HEXB* corrected the Hex activity deficiencies in the fibroblasts derived from patients with Tay-Sachs disease (α-deficiency) and Sandhoff disease (β-deficiency), respectively, and also eliminated the GM2 accumulated in the cells. However, administration of the murine counterparts to the human Tay-Sachs and Sandhoff disease fibroblasts, respectively, restored the Hex activities toward artificial substrates but had little effect on GM2 degradation. Introduction of the *HEXB* gene to the fibroblastic cell line derived from the *hexb*-disrupted mice (Sandhoff disease model; SD mice) did not also cause intracellular GM2 degradation, although a significant increase in the HexB activity toward neutral substrate was observed. In contrast, the conditioned media from the CHO cell lines simultaneously expressing *hexa* and *hexb* not only restored the intracellular HexA activity in a mannose-6-phosphate receptor-dependent manner, but also degraded the accumulated GM2 in the fibroblasts derived from the patients. In addition, the co-introduction of the *HEXA* and *HEXB* genes into the fibroblastic cell line derived from the SD mice caused significant degradation of the GM2. ÅyDiscussionÅzThese results suggested that human and murine α- and β-subunits could hardly associate with the xeno-counterparts to form a stable chimeric Hex A (αβ) in the cells. The constructed HexA models predicted the different amino acid residues between the human and murine β-subunits might involve in the α-β heterodimeric interaction. However, allo-Hex A could possibly interact with xeno-GM2 activator protein for GM2 degradation, suggesting that concomitant gene transfer of *HEXA* and *HEXB* into patients and receptor-mediated recombinant allo-HexA replacement could be applicable as therapies for GM2-gangliosidoses.

(411) **Comparison of Ion Exchange/Immunoassay with Immunoaffinity - Mass Spectrometry in Determination of Underglycosylated Transferrin in Alcohol Dependent Patients.**

John F. O'Brien[1], Jean M. Lacey[1], Daniel K. Hall-Flavin[2] and Gabrielle J. Melin[2]

[1] Department of Laboratory Medicine & Pathology, [2] Department of Psychiatry, Mayo Clinic, Rochester, MN 55905.

Background: Carbohydrate deficient transferrin (CDT) has been the most frequently mentioned new marker for alcoholism in the past two decades. Transferrin (Tr) is a glycoprotein synthesized in the liver and is the major iron carrier protein in blood. The apoprotein is a glycoprotein of 79560 kilodaltons (kDa) with two specific iron binding sites and two asparagine linked oligosaccharide moieties. Early studies on CDT used the protein resolution method of isoelectric focusing. Alcoholic patients have an increased proportion Transferrin (Tf) with an isoelectric point (pI) of > or = 5.7 in their sera. Due to the shift toward neutrality, CDT was presumed to be transferrin lacking terminal sialic acids on the N-linked oligosaccharides. Methods based ionic charge support the original assumption of the structure of CDT as glycoforms missing 1-4 sialic acids.
More recently, the ion exchange resolution-immunoassay detection method referred to as %CDT TIA has gained favor due to its FDA acceptance. Here we compare an immunoaffinity-MS (MS) method originally proposed for detection of congenital disorders of glycosylation (CDG) to the %CDT TIA method in patients admitted to the alcohol dependency unit at the Mayo Clinic.
Methods: %CDT TECT and Immunoaffinity-MS was run on samples from 54 fully consented patients admitted to the drug dependency unit. . %CDT TIA was run using kits and methods as described by the manufacturer. The inline MS method was previously reported by our laboratory. To demonstrate the molecular mass of the components from the %CDT TIA kit that were eluted in the CDT fraction, the low Cl^- column effluent was concentrated by 30,000 mw cutoff concentrators and subjected to immunoaffinity-MS.
Results: The methods result in values that show significant correlation (r=.8) establishing the contention of qualitative identity of CDT measured by MS and %CDT TECT.† Using a upper reference limit of mono-glycosylated/di-glycosylated of .063 (nearly equivalent to 6.3 % of Tr) for the MS method and 2.6% as suggested by the manufacturer of the %CDT TIA kits, the call of positive or negative agreed in 80% of the samples. The MS method results in fewer positive results.
MS of the CDT fraction from the %CDT TECT columns clearly shows the non-retained transferrin to be mono-glycosylated. This reaffirms our original observations on the nature of the glycosylation defect. The a-glycosylated form was detected by MS when the %CDT TIA was three times normal.
Conclusion: Immunoaffinity-MS and %CDT TECT perform similarly. The lower positive rate by MS may suggest that it has less sensitivity. Alternatively, false positive results by the %CDT TIA method can occur due to ionic changes in transferrin other than in the oligosaccharide moiety. These include primary structural changes (< 150 amu by MS) and failure to consistently load transferrin with Fe+++. The immunoaffinity-MS method only measures apotransferrin so it does not suffer from variability in the completeness of the iron binding step. The MS identification of CDT

casts doubt on the importance of the literature discussion as to which sialo- forms constitute abnormal transferrin in alcoholics.

(412) **Abnormal Glycosylation of Immunoglobulin A1 (IgA1) and Altered Transport of the Protein and Binding to the Polymeric Immunoglobulin Receptor.**

Parastoo Azadi[1], Gerardo Alvarez-Manilla[1], Trina Abney[1] and Steve. N. Emancipator[2]

[1] Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA , [2] Case Western Reserve University, Department of pathology, Cleveland, OH, USA.

Abnormal glycosylation of immunoglobulin A1 (IgA1) has been implicated in the pathophysiological characteristics of IgA nephropathy, leading to failure of normal clearance mechanisms and deposition of serum IgA1. We are examining the influence of N- and O- linked glycosylation on the transepithelial transport of human IgA1 glycoprotein. Polarized epithelia actively transport this glycoprotein via the polymeric immunoglobulin receptor (pIgR). Treatment of the glycoproteins with various combinations of sialidase, ,-galactosidase and glucosaminidase alter the transport of the protein, and the binding to the polymeric immunoglobulin receptor (pIgR). Our goal has been to characterize the products of the enzymatic treatments we use to perform the transport and binding experiments, especially to learn the extent to which the enzymes remove the specific residues. We have used a combination of ,-elimination and peptide- N -glycosidase F digestions on the various IgA1 preparations to release the O- and N-linked glycans respectively. A combination of glycosyl composition analysis and MALDI-MS analysis of permethylated and released glycans was used to elucidate the structure of the attached carbohydrates. The aim of this study was to develop sensitive methods to effectively release and characterize both N and O-linked glycans of IgA 1 glycoprotein in small quantities. This work is part an overall efforts to study the mechanisms and pathophysiologic consequences of aberrant glycosylation of the human circulating protein and IgA1 nephropathy disease in humans.

(413) **Respiratory Syncytial Virus (RSV) G Glycoprotein-Induced Eosinophilia Requires IL-5, Eotaxin, and CD4+ T Cells: Illness is Dependent on CD4+ T Cells and Not Eosinophils**

Teresa R. Johnson and Barney S. Graham

40 Convent Drive MSC 3017, Building 40 Room 2504, Bethesda, MD 20892-3017.

RSV G glycoprotein has an unusual protein structure with little homology to any known viral protein and is more like mucinous glycoproteins, with greater than 60% of its mature weight contributed by O-linked carbohydrate moieties. RSV G, the viral attachment glycoprotein, is expressed on the surface of virus particles and infected cells. While a protein receptor for RSV G has not been identified, it was demonstrated that the G glycoprotein binds to cellular glycosaminoglycans (GAGs) and heparan sulfates and that exogenous GAGs inhibit virus binding and infection. Additionally, purified RSV G binds to surfactant proteins. These data suggest a potential role for G glycoprotein binding to cellular carbohydrate moieties in RSV pathogenesis. A secreted form of the protein is also produced within 6 hours post-infection due to a second methionine codon in the transmembrane region of the protein. RSV suppresses anti-viral immune responses and ciliary-beat frequency and mucociliary clearance. The mechanisms for these inhibitory actions have not been identified. However, due to the rapid production of a secreted form and structural similarity to the chemokine fractalkine, RSV G may play important roles in altering respiratory physiology and anti-viral immunity. Eosinophil recruitment and degranulation in the airways has been demonstrated during severe RSV disease in natural human and bovine infections and in animal models of pathogenesis. Thus, it has been hypothesized that eosinophils and their granular enzymes contribute to illness. We have shown that immunization with vaccinia virus expressing the secreted form of the G glycoprotein (vvGs) predisposes for severe disease typified by pulmonary eosinophilia and type 2 cytokine production following live RSV challenge. Furthermore, we have shown that the induction of these immune responses does not require IL-4. Therefore, we sought to determine the requirements for IL-5, CD4+ and CD8+ T cells, and eotaxin in the induction of RSV G-specific memory immune responses. Antibody depletion of CD4+ T cells at the time of immunization prevented the induction of IL-4, IL-13, and eotaxin post-challenge, diminished eosinophilia, and reduced the severity of illness. Conversely, depletion of CD8+ T cells had no effect on eosinophilia after RSV challenge. Administration of IL-5 neutralizing antibodies at the time of immunization significantly decreased eosinophilia after challenge. However, there was not a concomitant decrease in illness in vvGs-primed, anti-IL-5-treated mice following RSV challenge. Eotaxin-deficient mice immunized with vvGs had significantly less eosiniophilia post-RSV challenge, and viral clearance was delayed. However, illness was not decreased. Thus, IL-5 produced by CD4+ T cells and eotaxin are required for vvGs-induced eosinophilia following challenge. However, the recruitment of eosinophils is a side-effect of memory CD4+ T cell activation and does not directly cause illness. Furthermore, the eosinophils may in fact contribute to anti-viral immunity and play a role in viral clearance as has been demonstrated with the paramyxovirus pnemovirus of mice.

(414) **The Influence of L-Selectin on Hematogenous Metastasis**

Jennifer L. Stevenson[1], Heinz L‰ubli[2], Peter J‰oggi[2], Ajit Varki[1], Nissi M. Varki[1] and Lubor Borsig[2]

[1] Glycobiology Research and Training Center, University of California, San Diego, [2] Institute of Physiology, University of Zurich.

Hematogeneous metastasis of carcinomas is accompanied by aggregation of platelets and leukocytes around tumor cells, forming emboli that arrest in capillaries at distant sites. We have shown that P-selectin facilitates metastases by mediating platelet-tumor cell interactions, which are critical for metastasis (PNAS 98:3352-3357). We have also shown that L-selectin deficiency attenuates hematogenous tumor metastasis, even in T and B cell deficient animals (PNAS 99: 2193-2198). Thus, L-selectin on monocytes and/or neutrophils can influence the metastatic process. Here we pursue the mechanisms involved. Studies with L-selectin deficient mice indicate that the presence of activated neutrophils in sites of tumor embolization is L-selectin dependent, and that there appears to be a change in timing of the subsequent accumulation of monocytes in L-selectin-deficient mice. The neutrophils observed in tumor emboli in wild-type mice were almost entirely absent in L-selectin-deficient mice, and the recruitment of differentiated monocytes to the tumor emboli was delayed. The presence of leukocytes within tumor cell emboli in wild-type mice was maximal at 12 hours post-tumor cell injection and was sustained for another 24 hours. These observations support the involvement of these leukocyte subpopulations at a later stage in the metastatic process, after the initial platelet aggregation and embolus formation. Further support for this comes from observing the effects of single dose injections of heparin (which inhibit L-selectin binding to its ligands) at various time points after the initial injection of tumor cells (note that all our earlier studies used a single heparin injection 30 min prior to tumor cell injections). The maximum effect of these later injections of heparin is seen 6 hours post tumor cell injection in WT mice, with reduced metastatic ability, similar to that seen in L-selectin deficient animals. Heparin injections in L-selectin-deficient mice at these later time points did not change the already low incidence of metastases. Thus, the effects of a later dose of heparin can be best explained by L-selectin blockade. Is the involvement of L-selectin in the metastatic cascade mediated by leukocyte interactions with L-selectin ligands on tumor cells, or on the endothelial cells (sites where tumor cells eventually exit from the bloodstream)? Regarding the first possibility, L-selectin ligands are currently being identified in vivo by intravenously injecting mice first with tumor cells, and then with fluorescently-conjugated mouse L-selectin chimera, after which lung tissues are isolated and cryosections analyzed. With regard to the second possibility, we studied FucT-VII null mice, which are markedly deficient in selectin ligands. We observed a lower rate of metastasis from intravenously injected tumor cells (similar to that seen in L-selectin deficient mice). These results are also of clinical relevance, since heparin treatment leads to attenuation of metastasis even after the tumor cells have already been in the circulation for several hours. These results provide further insights into the mechanism of L-selectin action, demonstrate how leukocytes can facilitate metastasis and indicate that heparin therapy could be used to inhibit L-selectin interactions that influence metastasis.

(415) **Pre-organization of the Bioactive Conformation of Glycomimetics for Lectins from Psuedomonas aeruginosa Enhances Activity into the Nanomolar Range**

Arun Sarkar and John L. Magnani

GlycoMimetics Inc., 101 Orchard Ridge Dr., Suite 1E, Gaithersburg, MD 20878 USA.

Pseudomonas aeruginosa is an opportunistic pathogen in immunocompromised patients. This bacteria is the major cause of morbidity and mortality in patients with Cystic Fibrosis and is a common pathogen responsible for nosocomial infections, many of which result in death of the

patient. Two soluble lectins produced by this bacteria (PA-IIL and PA-IL) are associated with cytotoxic factors and their genes, lec B and lec A, respectively, are under quorum sensing control. Mitchell et al [Nature Structural Biology 9:919-921(2002)] described the bioactive conformation of the Lewis carbohydrate structures docked into the crystal structure of the PA-IIL lectin. The stacking of the fucose under the galactose is critical for the bioactive conformation. Glycomimetics of the Lewis structures were synthesized and screened for binding PA-IIL lectin. Solution conformations of the glycomimetics were determined by NMR spectroscopy and the interglycosidic NOE effects were used to determine the degree of stacking of the fucose under the galactose. Pre-organization of this bioactive conformation correlates with activity of the glycomimetic for the PA-IIL lectin with one compound displaying an IC50 value of 100 nM. The PA-IL lectin is reported to bind galactose residues. Epitope determination of this binding site was carried out by in vitro binding assays. The PA-IL lectin, in contrast. binds weakly to the B-Gal in Lewis carbohydrate structures and displays much greatly affinity to alpha galactose residues. Development of potent glycomimetic compounds based on the bioactive conformation of these lectins presents a novel therapeutic approach to this pathogen that is becoming increasingly antibiotic resistant.

(416) **Prostaglandine E2 is a Potent Activator of HAS1 Transcription: The NSAID sodium salicylate acts as specific HAS1 Inhibitor by Blocking Cyclooygenase Activity**

Karl M. Stuhlmeier and Christine Pollaschek
Kurbadstrasse 10, Vienna, Austria.

Background: Very little is known about differences in function and pathways of activation of the three genes encoding hyaluronic acid (HA). We demonstrated earlier, that out of these genes, fibroblast-like synoviocytes (FLS) respond to an array of proinflammatory mediators nearly exclusively with the activation of HAS1. This points at HAS1 as a possible target for drug mediated intervention in a disease such as rheumatoid arthritis (RA) that is associated with and characterized by unfettered HA release. Understanding mechanisms and mediators of gene activation might offer new insight into gene functions. With these concepts in mind, we tested a series of drugs that have been successfully used and administered for years to patients to slow or prevent progression of rheumatic diseases. Here we report our findings on the effect of sodium salicylate and IL-1fl on HAS genes in FLS. Methods: FLS, isolated from RA patients and grown to high density in tissue culture dishes, were treated with sodium salicylate (0.5 and 5 μM respectively) followed by exposure to IL-1fl, PMA, and PGE2 for 6 to 24 hours. HA specific ELISA were used to assess HA levels in FLS culture supernatant. HA-synthase assays were utilized to measure salicylate effects on HA-synthase activity. RT-PCR was utilized to measure mRNA levels of the three HA genes and electrophoretic-mobility-shift-assays (EMSA) were used to test salicylate's effects on the transcription factor NF-kB. Results and Discussion: While mRNA for the gene HAS1 is very low or undetectable in quiescent FLS, the genes HAS2 and HAS3 are constitutively expressed. More importantly, mRNA levels of HAS2 and HAS3 are hardly affected by stimulation with a series of pro-inflammatory cytokines, but FLS respond to IL-1fl treatment with a dramatic increase in HAS1 mRNA transcription and release. RA is characterized by unfettered HA production and salicylate is widely used to treat RA. Tests were performed to see whether any of the HAS genes is affected by salicylate treatment of FLS and what mechanisms might account for such effects. Tests revealed that salicylate blocks IL-1fl induced HA release in a dose dependent manner. HA-synthase assays demonstrate reduced activity and RT-PCR showed dose dependent inhibition of HAS1 mRNA pointing at inhibition of transcription by salicylate. NF-kB is a trancription factor essential for the upregulation of many pro-inflammatory genes and salicylate is known to block the translocation of NF-kB. We demonstrate that at the doses used to block IL-1fl induced HAS1 mRNA, salicylate had no effect on the activation of NF-kB and the high doses reportedly used to block NF-kB resulted in cell damage. As salicylate blocks COX activity, we restored the COX pathway using exogenous PGE2 and found PGE2 a potent activator of HAS1. Further tests using a series of specific COX inhibitors revealed that cyclooxygenase products are essential for IL-1fl induced HAS1 upregulation and that salicylate prevented induced HAS1 activation by blocking both COX forms. The presented data offer new insight into the regulation of HAS1 and might help to explain some of the beneficial effects of salicylate.

(417) **Determination of the Antithrombin-binding Structure that is Generated by Heparan Sulfate 3-O-sulfotransferase-5**

Jinghua Chen and Jian Liu
Division of Medicinal Chemistry and Natural Products, School of Pharmacy, UNC-CH, Chapel Hill, NC 27599.

The 3-O-sulfation of glucosamine is a key modification step during the biosynthesis of anticoagulant heparan sulfate (HS). Both heparan sulfate 3-O-sulfotransferase-1 (3-OST-1) and 3-O-sulfotransferase-5 (3-OST-5) transfer sulfate to 3-OH group of glucosamine to generate antithrombin-binding heparan sulfate (HSact). Our previous study has shown that 3-OST-5 has unique substrate specificity as evidenced by the fact that 3-OST-5 generates both an antithrombin-binding site to exhibit anticoagulant activity and a binding site for herpes simplex virus 1 glycoprotein D to serve as an entry receptor for herpes simplex virus. Here we reported the isolation and characterization of the anticoagulant heparan sulfate oligosaccharides specifically generated by 3-OST-5. 3H-labeled heparan sulfate from CHO cells was exhaustively modified by 3-OST-1 to remove the 3-OST-1 modification sites completely. The anticoagulant inactive pool of 3-OST-1 pre-treated HS was further modified by 3-OST-5 to generate additional antithrombin-binding HS, which was designated as 3-OST-5-HSact. The resultant 3-OST-5-HSact was digested by nitrous acid (pH=4.5) followed by fractionation on Biogel P-6. We found that 3-O- [35S] sulfated oligosaccharides (generated by 3-OST-5) were present in greater than octasaccharides. This observation suggests 3-OST-5-HSact is located within a domain clustered with N-sulfated glucosamine units. Additional structural analysis revealed that 3-OST-5-HSact oligosaccharide were found to be highly sulfated and composed of tri-sulfated disaccharides and tetra-sulfated disaccharides. The results from this study indicate that 3-OST-5 generates unique structures that bind to antithrombin.

(418) **Increased O-GlcNAc in Response to Stress, a Survival Response of Mammals**

Natasha E Zachara[1], Steven P Jones[2], Eduardo Marban[2] and Gerald W Hart[1]
[1] Deptartment of Biological Chemistry, The Johns Hopkins Unicersity School of Medicine, [2] Institute for Molecular Cardiology, The Johns Hopkins University School of Medicine.

Myriad nuclear and cytoplasmic proteins are modified by monosaccharides of O-linked β-N-acetylglucosamine, an essential and dynamic post-translational modification of metazoans. In response to multiple forms of cellular stress, levels of O-GlcNAc are rapidly and dynamically elevated on multiple proteins. Previously, we have shown that modulation of O-GlcNAc levels (both genetically and pharmacologically) increased the tolerance of cells to heat stress. Conversely, decreasing O-GlcNAc levels reduces stress tolerance. O-GlcNAc mediated stress tolerance appears to be linked to the synthesis of heat shock proteins, which have previously been shown to be protective in many in vitro and in vivo stress tolerance models. We have extended this study to examine the role of O-GlcNAc in cardiomyocytes treated with lethal doses of oxidative stress, a model of ischemia reperfusion injury. Raising O-GlcNAc levels in primary rat cardiomyocytes, using PUGNAc (an inhibitor of O-GlcNAcase), results in cells that are more resistant to oxidative stress. Interestingly, after cells become Annexin V positive (a marker of apoptosis) levels of O-GlcNAc declined globally. Notably, this is associated with the formation of a high molecular weight complex of the O-GlcNAc transferase that appears to be less active. To extend this study into a more clinically relevant model, levels of O-GlcNAc were raised in mice that were then subjected to left coronary artery occlusions. Mice treated with saline vehicle (n=14), PUGNAc (n=8; 50 mg/kg), or acute ischemic preconditioning (IPC; n=8) were then subjected to 40 minutes of in vivo left coronary artery occlusion followed by 24 hours of reperfusion. At the end of reperfusion, the hearts were stained for infarct size analysis. PUGNAc induced a 30% reduction ($p<0.05$) in myocardial infarct size compared to vehicle. Interestingly, in mice subjected to ischemic preconditioning, using either a classic or delayed model, O-GlcNAc levels are elevated when normalized to sham (n=6/group); classic (141+/-20%) or the delayed mode (137+/-7%). Taken together, these data suggest that O-GlcNAc is a key post-translational modification employed by cells and whole animals to rapidly respond to and survive stress. Our findings open a new avenue of research for our greater understanding of disease states associated with stress response pathways, in particular Ischemica-reperfusion injury. Supported by HD13563 and CA42486 to GWH, and the NHLBI NIH contract No. N01-HV-28180. Under a licensing agreement between Covance Research Products and The Johns Hopkins University, Dr. Hart receives a share of royalty received by the university on sales of the CTD 110.6 antibody.

(419) **Galectin-3 Deficient Macrophages Exhibit Defective Cell Migration**

Daniel K Hsu, Alexander I Chernyavsky, Sergei A Grando and Fu-Tong Liu

Department of Dermatology, University of California Davis Medical Center, Sacramento, CA, United States, 95817.

The endogenous animal lectin, galectin-3, is a galactose binding lectin and is expressed in monocytes/macrophages. Galectin-3 is involved in diverse functions, including regulation of the cell cycle and cell growth, apoptosis and RNA processing. In order to facilitate identification of cellular processes involving galectin-3, we generated a galectin-3 null (gal3-/-) mouse in the C57BL/6 background. In earlier studies, we observed decreased levels of chemically-induced peritoneal inflammation in these mice. In particular, significantly reduced numbers of infiltrating macrophages were observed, and this was a major factor in reduced levels of inflammation in gal3-/- mice. Additional studies with bone marrow derived macrophages (BMMΦ) revealed that cells from gal3-/- mice exhibited defects in phagocytosis. In gal3-/- BMMΦ cells undergoing phagocytosis, defective F-actin formation was observed. Therefore, we considered possible impairment of other F-actin dependent processes in these mice. We now show that BMMΦ are defective in chemotaxis to macrophage chemotactic protein-1 (MCP-1) and monocyte/macrophage colony stimulating factor-1. BMMΦ also demonstrate impaired general migration when cultured in an agarose outgrowth system. Under conditions of simulation with fMLP or MCP-1, galectin-3 was found to co-localize with F-actin at the base of the cells, and also colocalizes with F-actin and vinculin at podosomes. In an in vivo air pouch invasion assay, delayed chemotactic migration of gal3-/- macrophages to MCP-1 were also observed. Our results suggest that galectin-3 plays a significant role in adaptive and innate immunity by contributing to migration of macrophages.

(420) Effects of Leflunomide on Hyaluronan Synthases: Nuclear-factor-kappa-B Independent Suppression of interleukin-1 induced HAS1 Transcription by Leflunomide

Karl M. Stuhlmeier.

Kurbadstrafle 10, Vienna, Austria Kurbadstrafle 10, Vienna, Austria.

Hyaluronic acid (HA) is essential in many physiological mechanisms. Nevertheless, unfettered HA release is also associated with diseases such as rheumatoid arthritis. Hyaluronan synovial effusions e.g. serve as a sensitive indicator of altered connective tissue cell function, associated with inflammation. Elevated HA levels in serum can also serve as marker for rheumatoid arthritis (RA) as HA levels in RA patients are elevated to a degree that these can be readily detected. Many reports also demonstrate innumerable pro-inflammatory effects of hyaluronan fragments resulting from unregulated HA release. We hypothesized that uncontrolled hyaluronan release might contribute to genesis and progression of rheumatoid arthritis. Recently published data demonstrating that HA injection does indeed induce RA supports such a concept. We demonstrated earlier that fibroblast-like synoviocytes (FLS) release large amounts of HA in response to most pro-inflammatory cytokines. We further demonstrated that FLS respond to pro-inflammatory cytokines with the activation of HAS1, while HAS2/3 are mostly unaffected. Here we investigate effects and mechanisms of leflunomide on HAS genes. Leflunomide is a powerful immunomodulating drug that is approved for and successfully used in the treatment of RA. † FLS, isolated from RA patients, were treated with 5 and 50 µM leflunomide respectively followed by exposure to IL-1alpha, IL-1beta and PMA for 6 to 24 hours. HA specific ELISA were used to assess HA levels in culture supernatant. HA-synthase assays were utilized to measure leflunomide effects on HA-synthase activity. RT-PCR was utilized to measure mRNA levels of the three HA genes and electrophoretic-mobility-shift-assays (EMSA) were used to test leflunomide's effects on the transcription factor NF-kB. Treatment with leflunomide significantly decreased IL-1alpha, IL-1beta and PMA induced HA levels in culture supernatant in a dose dependent manner. The observed reduction in HA levels is due to inhibition of HA-synthase activity, since HA-synthase assays revealed a dose dependent inhibition by leflunomide. Further experiments, stimulating FLS with IL-1beta or PMA, demonstrated the gene specificity of this drug in that only mRNA levels of HAS1 were affected. Leflunomide has been shown to inhibit the activation and translocation of NF-kB, a transcription factor of utmost importance in the activation of most pro-inflammatory genes. Our data demonstrate that IL-1beta is a powerful activator of NF-kB in FLS. Nevertheless, leflunomide at concentrations that completely blocked IL-1 induced HAS1 mRNA transcription had absolutely no effect on the activation of NF-kB, excluding this well recognized mechanism as the mode of action of leflunomide on IL-1 induced HAS1 activation. Furthermore, restoring the known suppressive effect of leflunomide on pyrimidine syntheses using exogenous uridine was also without effect. Leflunomide was ascribed kinase inhibitor activity as well. We therefore tested a series of kinase-inhibitors and found that specific tyrosine kinase inhibitors can block IL-1beta induced HAS1 mRNA transcription - an effect mimicked by leflunomide. These data indicate the importance of tyrosine kinases in the activation pathway of IL-1beta induced HAS1 activation and might point at novel ways of preventing undesired HAS1 activation. In addition, these findings might also explain some of the drug effects in the treatment of RA.

(421) ***Cicer arietinum* Lectin Specific Annexin II Translocation to the Cell Surface and Role in Epithelial Wound Repair**

Benjamin J. Patchell and Delbert R. Dorscheid

University of British Columbia/ James Hogg iCAPTURE Centre, Department of Experimental Medicine, Vancouver, BC Canada.

Airway epithelial repair is an essential function in the restoration of tissue integrity following daily challenges. The epithelium of asthmatics is chronically damaged and locked in a repair phenotype. N-glycosylation has previously been shown to be important in the migration of epithelial cells. We have previously identified changes in the surface glycosylation on damaged epithelial areas. Our laboratory has identified *Cicer arietinum* lectin (CPA) as a lectin that differentially stains epithelial cells in damaged areas of the epithelium relative to intact epithelium. We hypothesize that CPA could be used as a marker to identify functional glycoproteins involved in the normal repair of wounded epithelial monolayers. Wounded airway epithelial cell (1HAEo$^-$) monolayers were either stained with or exposed to CPA added to the media to characterize a role for CPA specific carbohydrate structures in repair. CPA-identified glycoproteins were purified by lectin precipitation and sequenced by MALDI-TOF mass spectrometry. Subsequent cell surface protein biotinylation and immuno-staining was used to detect the translocation of Annexin II to the cell surface and its presentation at the leading edge of the wound respectively in our airway epithelial wound repair model. Following mechanical injury, CPA staining was restricted to the leading edge of the wounded monolayers. The addition of CPA to the culture media immediately after wound creation resulted in no inhibition of wound closure. However; when CPA was added 6 and 12 hours after wound creation, wound closure was inhibited in a dose dependant manner. The percentage of remaining wound area 24 hours after mechanical wounding was calculated: negative control 42 ± 12%, EGF stimulated 9 ± 4%, co-treatment with EGF and increasing doses of CPA; CPA 10 µg/ml 6 ± 2%, CPA 25 µg/ml 13 ± 5%, CPA 50 µg/ml 20 ± 4% and CPA 100 µg/ml 32 ± 10%. These results suggest a novel cell surface glycoprotein ligand is presented shortly (~6 hrs) after the creation of the wound. Following precipitation by CPA and MALDI-TOF sequencing, Annexin II (AII) was identified as the candidate glycoprotein effecting repair. By membrane protein biotinylation, cell surface AII was detected 6 hrs after mechanical wounding. These findings were confirmed by AII immunostaining, cell surface expression appears to be restricted to those cells adjacent to the edge of mechanically created wounds. Our findings suggest AII is glycosylated or part of a CPA specific glycosylated complex that is translocated to the cell surface. The translocation of AII appears to facilitate the migration and repair of damaged airway epithelium acting almost entirely at the leading edge of the wound.

(422) ***In vitro* and Fibroblast Culture Measurements of UDP-GlcNAc 2-epimerase/ManNAc Kinase Activity in Hereditary Inclusion Body Myopathy**

Susan E. Sparks[1], Carla Ciccone[1], Molly Lalor[1], Eduard Orvisky[2], Donna Krasnewich[1], M-S Sun[1], William A. Gahl[1] and Marjan Huizing[1]

[1] Section of Human Biochemical Genetics, MGB, NHGRI, NIH, Bethesda, MD, [2] NSB, NIMH, NIH, Bethesda, MD.

UDP-GlcNAc 2-epimerase/ManNAc kinase, encoded by *GNE* on chromosome 9p12-13, is the bifunctional and rate-limiting enzyme in the biosynthesis of sialic acid. The epimerase converts UDP-GlcNAc to ManNAc and the kinase converts ManNAc to ManNAc-6-phosphate. Mutations in either or both the epimerase and kinase domains of *GNE* cause Hereditary Inclusion Body Myopathy (HIBM), an autosomal recessive disorder characterized by adult-onset, progressive distal and proximal muscle weakness sparing the quadriceps muscles. Cultured fibroblasts and a

cell-free transcription/translation system were employed to investigate the effects of individual *GNE* mutations of 3 HIBM patients on the epimerase and kinase activities. The epimerase activity was determined by measuring the conversion of ^{3}H-UDP-GlcNAc to ^{3}H-ManNAc. The conversion rate of ^{3}H-ManNAc to ^{3}H-ManNAc-6P was used for the kinase activity. *GNE* was cloned into a pET17b expression vector and a patient's specific *GNE* mutations were created by site-directed mutagenesis. The resulting proteins were subsequently translated in the cell-free system and the epimerase and kinase activities were measured. The cell-free system allows measurement of GNE activity without influences from other enzymes present in fibroblasts. In the cell-free system, the HIBM mutant GNE-epimerase activity was negligible compared to wild type GNE. In cultured fibroblasts from HIBM patients, residual epimerase activity ranged from 38% to 83% of normal. We conclude that fibroblasts contain enzymes, besides GNE, that convert UDP-GlcNAc to ManNAc. When the Iranian Jewish founder mutation, 2186T>C (M712T), was cloned into *GNE* and expressed in the cell-free system, the GNE-kinase activity was 28% of normal. Cultured fibroblasts from an HIBM patient with a homozygous 2186T>C mutation had 26% GNE-kinase activity compared to normal fibroblasts. This indicates that fibroblasts do not contain other enzymes that convert ManNAc to ManNAc-6P. In addition, the M712T mutant GNE itself exhibits residual ManNAc kinase activity. Comparison of GNE epimerase and kinase activity in both systems will help to elucidate the underlying basis of the widely variable phenotypes of HIBM patients with specific mutations in the epimerase or kinase domains.

(423) **Incorporation of the Non-human Sialic Acid Neu5Gc into Human Leukemic Cells and Targeting by Natural Human Anti-Neu5Gc Antibodies**

Dzung H. Nguyen, Pam Tangvoranuntakul and Ajit Varki
Glycobiology Research and Training Center and Department of Cellular and Molecular Medicine, University of California, San Diego.

Humans are incapable of synthesizing the common mammalian cell surface sialic acid N-glycolylneuraminic acid (Neu5Gc), due to an irreversible genetic mutation that occurred after our last common ancestor with great apes. Despite this, we found trace levels of Neu5Gc in certain normal human tissues, and higher levels in fetal and malignant tissues, apparently due to incorporation from dietary sources. Clinicians have known for decades that many cancer cell types express Hanganutziu-Deicher (HD) antigens, which have since been identified as Neu5Gc-bearing glycoconjugates. Interestingly, circulating anti-Neu5Gc antibodies occur in most normal humans, with marked individual variations in levels, while certain disease states, such as infectious mononucleosis, rheumatoid arthritis, syphilis, and leprosy, are associated with elevated levels of antibodies against Neu5Gc. In a small study using a sensitive ELISA, we could detect measurable amounts of antibodies in at least 2 out of 3 Ig subtypes, IgG, IgA or IgM, in all 21 healthy subjects. We now show that while normal human blood cells metabolically incorporate very little free Neu5Gc from the culture medium, human leukemic cell lines do so efficiently, displaying significant levels on their cell surfaces within 2 to 4 hours of incubation, as detected by flow cytometry using a monospecific polyclonal chicken antibody, and confirmed chemically by HPLC. This Neu5Gc incorporation into leukemic cells occurred in a concentration-dependent manner. Specific deposition of IgG from Neu5Gc-reactive human sera onto Neu5Gc-expressing leukemic cells was observed. This was associated with lytic cell killing, apoptosis, and antibody-dependent cell-mediated cytotoxicity. Human sera with low levels of anti-Neu5Gc IgG did not mediate such effects. These data show for the first time that the 'natural' anti-Neu5Gc antibodies found in normal humans can be functionally active. The selective incorporation of Neu5Gc into leukemic cells could provide an approach for targeting leukemic cells in vivo, via naturally occurring Neu5Gc-specific antibodies. However, in contrast to unstimulated blood cells, activated human T-lymphocytes also incorporated some Neu5Gc, albeit to a lesser degree than leukemic cells, allowing IgG binding and complement deposition. Thus, exposure of rapidly dividing activated T cells to Neu5Gc could potentially affect ongoing immune responses. Since incorporation into leukemic cells was most efficient and cancer cells tend to be hyper-sialylated compared to normal cells, it may still be possible to define a therapeutic window to selectively target malignant cells.

(424) **Neu4, a Novel Human Lysosomal Lumen Sialidase Confers Normal Phenotype to Sialidosis and Galactosialidosis Cells.**

Alexey V. Pshezhetsky[1], Volkan Seyrantepe[1], Karine Landry[1], Stephanie Trudel[1], Jacob A. Hassan[2] and Carlos R. Morales[2]
[1] Hopital Sainte-Justine and Departement de pediatrie, Universite de Montreal, Montreal, Quebec H3T 1C5 Canada , [2] Department of Anatomy and Cell Biology, Faculty of Medicine, McGill University, Montreal (Quebec), H3A 2B2 Canada .

Three different mammalian sialidases: lysosomal (Neu1, gene NEU1), cytoplasmic (Neu2, gene NEU2) and plasma membrane (Neu3, gene NEU3) have been described. Inherited deficiency of Neu1 in humans, due to mutations in the NEU1 gene causes severe multisystemic neuradegenerative disorder, sialidosis. Clinically similar disorder, galactosialidosis is caused by the secondary Neu1 deficiency due to genetic defects in cathepsin A that forms a complex with Neu1 and activates it. In this study we describe a novel lysosomal lumen sialidase encoded by the NEU4 gene on human chromosome 2. We demonstrate that Neu4 is ubiquitously expressed in human tissues and has broad substrate specificity being active against sialylated oligosaccharides, glycoproteins and gangliosides. In contrast to Neu1, Neu4 is targeted to lysosomes by the mannose-6-phospate receptor and does not require association with other proteins for enzymatic activity. Expression of the Neu4 in the cells of sialidosis and galactosialidosis patients results in clearance of storage materials from lysosomes suggesting that Neu4 may be useful for developing new therapies for these conditions.

(425) **Proposed Molecular Mechanisms of Protein-Losing Enteropathy**

Lars Bode[1], Camilla Salvestrini[2], Simon Murch[2], Pyong W Park[3], Robert J Linhardt[4] and Hudson H Freeze[1]
[1] The Burnham Institute, Glycobiology and Carbohydrate Chemistry Program, La Jolla, CA, USA, [2] Center for Pediatric Gastroenterology, Royal Free and University College Medical School, London, UK, [3] Department of Medicine, Baylor College of Medicine, Houston, TX, USA , [4] Rensselaer Polytechnic Institute, Troy, NY, USA .

Protein-losing enteropathy (PLE), the loss of plasma proteins through the intestine, is a life-threatening symptom in a number of ostensibly unrelated diseases, e.g. Congenital Disorders of Glycosylation (CDG), Inflammatory Bowel Diseases (IBD), or as a late complication after Fontan surgery to correct congenital heart defects. Despite the diversity of these disorders, PLE shares several common features: PLE is episodic and its onset is often associated with a proinflammatory state. During episodes of PLE heparan sulfate (HS) proteoglycans (HSPG) are absent specifically from the basolateral surface of small intestinal epithelial cells. HSPG return when PLE resolves. Our objective is to identify the key players involved in the molecular mechanisms of protein leakage. Recently, we established a tissue culture model of PLE-like protein leakage by studying the flux of FITC-labeled albumin through a monolayer of HT29 cells, an intestinal epithelial cell line. Our results showed that loss of cell-associated HS directly causes protein leakage, but also greatly amplifies protein leakage induced by the pro-inflammatory cytokine TNFα. The addition of soluble HS or heparin compensates for loss of cell-associated HS and alleviates TNFα-induced protein leakage in a concentration-dependent manner. Heparin alleviates TNFα-induced protein leakage only when co-incubated with cells suggesting a direct interaction between heparin and TNFα. The mitigating effects of heparin and heparin-like molecules help to explain the favorable response of some post-Fontan patients to subcutaneous injections of high molecular weight heparin. However, unfractioned heparin is not ideal, since it can cause osteoporosis or bleeding. Therefore, we screened several other glycosaminoglycans and oligosaccharides to identify the most potent compound without these undesirable side effects. The Fontan procedure often causes venous hypertension. Using a similar tissue culture approach, we showed that increased pressure alone causes protein leakage, and that loss of basolateral cell-associated HS amplifies pressure-induced protein leakage. Our results identify loss of HS, TNFα, and increased pressure as key players in the molecular pathology of PLE. Moreover, these factors combine to cause tremendous protein leakage. To extend our in vitro data, we aim to establish a mouse model of PLE. Preliminary results show that mice genetically deficient in syndecan-1 have increased intestinal protein loss as determined by fecal α-1-antitrypsin. These findings, along with the fact that syndecan-1 is predominantly expressed on the basolateral surface of epithelial cells, suggests that syndecan-1 is the HSPG affected in PLE. Further experiments will examine the role of TNFα, other pro-inflammatory cytokines, and increased pressure leading to PLE in vivo. This may help to guide therapeutic studies using heparin and heparin-like molecules to treat or even prevent PLE. Supported by the Children's Hearts Fund and the Deutsche Forschungsgemeinschaft (DFG BO 2488/1-1). Dedicated to the memory of Violet Niles and Colin Colson.

(426) **Monosaccharides Provide an Efficient Delivery Method for Butyrate, a Short Chain Fatty Acid (SCFA) with Anti-cancer Activity**

Mark B Jones, Srinivasa-Gopalan Sampathkumar and Kevin J Yarema
Whitaker Biomedical Engineering Institute, The Johns Hopkins University, 3400 N Charles St, Clark Hall Rm 107, Baltimore; MD 21218.

††††††††††The introduction of exogenously-supplied sugar analogs into cellular metabolic pathways has many potential biotechnological and biomedical applications.† For instance, important sugar-based medicines now under consideration include antibiotics, anti-cancer drugs, and anti-viral agents.† Unfortunately, sugar analogs are not 'drug-like' as they are often taken up by the target cells with low efficiency, often requiring concentrations of 25-50 mM to achieve a measurable response.† By contrast, several studies have shown that per-acetylated simple sugars are used with a several hundred-fold increase in efficiency.† We recently demonstrated that increasing the hydrophobicity of hydroxyl-protecting esters by extending the N-acyl chain length further improved the metabolic efficiency of N-acetylmannosamine (ManNAc) analog up-take by a cell and subsequent incorporation into the sialic acid biosynthetic pathway (J. Biol. Chem. **279**, 18342-18352 (2004)). †A drawback of this approach was that the longer N-acyl chain size rendered the analogs toxic in several human cell lines.†

††††††††††A mechanism was indicated by the progressive increase in toxicity observed for sugars bearing acetyl, propanoyl, and butanoyl protecting groups.† Once inside the cell non-specific esterases hydrolyze these groups from the core sugar thereby generating the respective short chain fatty acids (SCFA).† Interestingly, SCFAs also are generated in the digestive system by symbiotic fauna and protect against gastrointestinal cancer by selectively inducing apoptosis in pre/early-stage cancerous cells while avoiding harm to healthy cells.† SCFAs trigger the expression of certain sets of genes in treated cells allowing the $p21^{WAF1/CIP1}$ promoter to be used diagnostically to detect SCFA effects.† In our assays AD293 cells were transiently transfected with a plasmid containing the $p21^{WAF1/CIP1}$ promoter element linked to a luciferase gene.† The cells subsequently were incubated with butyrate-derivatized ManNAc analogs (or sodium butyrate, NaBT), lysed and assayed for luciferase activity.† The results show that monosaccharide analog-derived butyrate activated the $p21^{WAF1/CIP1}$ promoter at ~1% of the concentration required for NaBT. †By contrast, sugar-derived acetate, propionate, and valerate showed weak if any activation in the luciferase assay. †These results are consistent with many published studies that report that butyrate has much stronger anti-cancer (and other SCFA-related) properties than either shorter or longer SCFAs. †These findings are significant because butyrate is not a clinical option for most cancers because of the high concentrations (~10 mM) required for efficacy; consequently simple sugars present an attractive alternative as efficient and safe delivery vehicles.

(427) **Sulfated High-Mannose Type Oligosaccharides in Cruzipain, the Major Cysteine Proteinase of *Trypanosoma Cruzi*.**

Mariana Barboza [1], Vilma G. Duschak [2], Yuko Fukuyama[3], Hiroshi Nonami[3], Rosa Erra-Balsells[4], Juan J. Cazzulo [1] and Alicia S. Couto [4]
[1] IIB-INTECH, UNSAM-CONICET, Argentina, [2] Instituto Nacional de Parasitolog'a, Dr. Mario Fatala ChabÈn, Ministerio de Salud, Argentina, [3] College of Agriculture, Ehime University, Japan, [4] CIHIDECAR-Departamento de Qu'mica Org·nica, FCEyN, UBA, Argentina.

Trypanosoma cruzi, the parasitic protozoan which causes Chagas disease, the American Trypanosomiasis, contains a major cysteine proteinase, cruzipain. This lysosomal enzyme bears an unusual carboxy-terminal extension (C-T) that contains a number of post-translational modifications and most antibodies in natural and experimental infections are directed against it. In the present work we report the presence of sulfated N-linked oligosaccharides as components of a lysosomal glycoprotein of the parasitic protozoan *Trypanosoma cruzi*. We took advantage of UV-MALDI-TOF mass spectrometry in conjunction with peptide N-glycosidase F deglycosylation and high performance anion exchange chromatography analysis to address the structure of the N-linked oligosaccharides present in the C-T domain. The UV-MALDI-TOF MS analysis in the negative-ion mode, using nor-harmane as matrix, allowed us to determine a new striking feature in cruzipain: sulfated high-mannose type oligosaccharides. Sulfated $GlcNAc_2Man_3$ to $GlcNAc_2Man_9$ species were identified. Interestingly, the signal at m/z 990 corresponding to the sulfated core structure strongly indicated that the sulfate group is linked to a core-GlcNAc. Futhermore, by UV-MALDI-TOF mass spectrometry analysis in the positive-ion mode showed (i) a main population of high-mannose type oligosaccharides, (ii) lactosaminic glycans were also identified, among them, structures corresponding to monosialylated species and (iii) as an interesting fact, fucosylated oligosaccharides were also characterized. The presence of the deoxy sugar was further confirmed by high performance anion exchange chromatography. In conclusion, the total number of oligosaccharides occurring in cruzipain showed to be much higher than estimated before. Sulfated N-linked-oligosaccharides have been reported as components of glycoproteins from viruses, mammalian cells, birds and a few other organisms. Sulfated high-mannose type glycans have only been described as component of glycoproteins from *Dictyostelium discoideum*. Likewise, these glycoproteins are localized in lysosome, however, Man-6-SO_4 accounts for the majority of the sulfated sugar. In *Trypanosoma cruzi*, sulfated structures have been described as part of glycolipids. The present study constitutes the first report on the presence of sulfated oligosaccharides in glycoproteins of *T. cruzi*. The finding of sulfated glycans indicates the presence of an active sulfotransferase which has not been described in *Trypanosoma cruzi* so far. Studies to address the precise nature of the sulfate sustitution and their biological significance are currently in progress in our laboratory.

(428) **The role of COG complex in Intracellular Trafficking, Golgi functions, Cell Morphology, and Development in Humans**

Xiaohua Wu, Ognian Bohorov and Hudson Freeze
The Burnham Institute, 10901 N. Torrey Pines Road, La Jolla, California 92037, USA..

Conserved Oligomeric Golgi (COG) complex associates with the cytosolic surface of the Golgi. It consists of 8 subunits, and is thought to be a tethering factor that plays an important role in intracellular trafficking and the structure/functions of the Golgi apparatus. The precise functions of the COG complex(s) and/or individual COG subunits remain unknown. Here, we present data at the molecular/cellular and organism levels revealing some of the functions of the COG complex. At molecular/cellular level, we studied COG1-deficient (ldlB) and COG2-deficient (ldlC) CHO cells and a COG7-deficient human dermal fibroblast. The mutations in COG1, 2 and 7 impair integrity of the COG complex and alters Golgi trafficking, resulting in the disruption of multiple glycosylation pathways. Our results showed that in all three mutant cell lines the activities of CMP-NeuAc and UDP-Gal transporters are reduced substantially, as well as the activities of ST3Gal-I and Core1GalT enzymes. Furthermore, using ST3-Gal-GFP and/or VSV-G-GFP proteins as markers, we demonstrated that the trafficking from ER to Golgi is altered in the mutant cells. We examined the 3D-structure of the Golgi apparatus and microtubules in control and COG mutant cells at 50-100 nm resolution. The Golgi structures, localization, microtubule organization, and cell morphology were altered dramatically in COG1-deficient cell and much less severely in COG2-deficient cell but there was no obvious defect in COG7-deficient cells. Introducing normal COG1, 2 and 7 cDNAs, respectively, restored the intracellular trafficking and Golgi structures, as well as glycosylation in three mutant cell lines. In humans, we discovered that two siblings harboring a homozygous point mutation in COG7 resulted in their death shortly after birth. Both patients had almost identical clinical presentations: perinatal asphyxia and dysmorphia including low-set, dysplastic ears, micrognathia, short neck, and loose wrinkled skin. Generalized hypotonia, hepatosplenomegaly, and progressive jaundice developed shortly after birth. X-ray examination showed that one patient lacked humerus and tibia epiphysis while other patient had short extremities. They developed severe epilepsy, failed to thrive, and died within 10 weeks after birth from recurrent infections and cardiac insufficiency. These cases represent a new type of Congenital Disorder of Glycosylation in which the molecular defect lies in a protein that affects the trafficking and function of the glycosylation machinery. (Supported by RO1DK55615)

(429) **Altered O-Glycosylation and Sulfation of Airway Mucins Associated with Cystic Fibrosis**

Baoyun XIA[1,2,3], Goverdhan P. Sachdev[2,3] and Richard D. Cummings[1,2]
[1] Department of Biochemistry & Molecular Biology, The University of Oklahoma Health Sciences Center Oklahoma City, OK 73104 , [2] Oklahoma Center for Medical Glycobiology, The University of Oklahoma Health Sciences Center Oklahoma City, OK 73104 , [3] College of Pharmacy, The University of Oklahoma Health Sciences Center Oklahoma City, OK 73104 .

Cystic fibrosis is an autosomal genetic disease resulting in the accumulation of mucins in exocrine organs. The disease results from mutations in the CFTR gene on chromosome 7 encoding the CFTR protein, which is a

member of the ATP binding cassette (ABC) family of ion transporters. The disease is common and 1 in 25 individuals of Northern European extraction carry a mutation in CFTR. A characteristic of cystic fibrosis is lung disease, resulting from excessive mucin production and accumulation and thickening of the mucus plaques and plugs. The accumulated mucin promotes bacterial adhesion and colonization, primarily *Pseudomonas aeruginosa*, resulting in increasing lung pathology and obstruction and eventual death. The estimated mean survival age of affected individuals as of 2001 was only 33.4 years. Previous studies have examined the glycosylation of airway mucins in patients suffering from cystic fibrosis (CF), but a systematic comparison of O-glycan structures in high-molecular weight mucins from multiple non-diseased (ND) versus CF individuals has not been reported. Here we describe our comparison of O-glycan structures in sputum-derived mucins from two ND donors and two CF patients. Analysis of all mucins indicated the presence of galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose (Fuc), and sialic acid (N-acetylneuraminic acid-NeuAc), but CF mucins had an overall higher sugar content with significant increases in NeuAc, Fuc, and GalNAc. O-glycans wee released from CF- and ND- mucins by ammonia-based procedures, derivatized with 2-aminobenzamide (2-AB), separated by ion exchange chromatography, and quantified by fluorescence. Glycan compositions were defined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). In general there was about a 50% increase in sialylation and sulfation in CF-mucin compared to ND-mucin. Unexpectedly, over 200 individual 2-AB-labeled O-glycans were identified by MALDI-TOF-MS, and the higher proportion of sialylated and sulfated O-glycans were apparent in the CF-mucin sample. Major differences in O-glycan structures were seen for CF- versus ND-mucins, demonstrating that both quantitative and qualitative changes are found in mucins from CF patients. Both ND and CF mucins contained GlcNAc-6-sulfate, Gal-3-sulfate, and Gal-6-sulfate, but CF mucins had significantly higher amounts of the 6-sulfated, but not the 3-sulfated residues. Such found structural difference in mucin glycosylation in CF patients may contribute to increased pathogenesis by *Pseudomonas aeruginosa* and fatal pneumonia.

(430) **Quantitative Analysis of β1,3-*N*-Acetylglucosaminyl Transferase and β1,4-Galactosyl Transferase mRNAs in Human Cornea.**

Kazuko Kitayama and Tomoya O. Akama
Glycobiology program, The Burnham Institute, La Jolla, CA, USA..

Keratan sulfate proteoglycans play important roles for maintaining of corneal transparency. So far, corneal *N*-acetylglucosaminyl 6-*O*-sulfotransferase and keratan sulfate galactose 6-*O*-sulfotransferase are responsible for sulfation of corneal keratan sulfate chain. However, the enzymes acting on elongation of the keratan sulfate carbohydrate chain in the cornea have not been identified yet. To understand the biosynthesis of keratan sulfate of human cornea, we analyzed mRNA expression of β1, 3-*N*-acetylglucosaminyl transferase (β3GnT) and β1,4-galactosyl transferase (β4GalT) genes, which are candidate enzymes for extension of keratan sulfate carbohydrate chain in the cornea by quantitative RT-PCR. All of the β4GalT mRNAs and β3GnT mRNAs except β3GnT6 mRNA was detected in the human cornea by the RT-PCR analysis. By quantitative RT-PCR analysis, we also found that β4GalT2, 3 and β3GnT7 mRNA expression levels were higher than those of other β4GalTs and β3GnTs, respectively. Our data suggests that various β4GalTs and β3GnTs, especially β4GalT2, 3 and β3GnT7, are involved in the extension of carbohydrate chain of keratan sulfate in the human cornea.

(431) **Fourier Transform Infrared Spectroscopy in the Characterization of Skin**

Maria O. Longas[1], Kenya Cheairs[1], Michelle L. Puchalski[1] and Jung I. Park[2]
[1] Purdue University Calumet, Department of Chemisltry and Physics, Hammond, IN 46323, [2] St. Margaret Mercy Health Care Center, Dyer, IN 46311.

Human skin may be classified mainly as Asian, Black or White. The difference in color is believed to arise from the content of the natural pigment, melanin. In this study we utilized Fourier Transform Infrared Spectroscopy (FT-IR) to demonstrate skin differences based on the absorption of infrared light. The reflectance diffusion technique was employed to analyze specimens from different subjects of each race group. FT-IR bands were quantified and the differences between the races were determined. The intensity of the IR band at 1727-1635 cm^{-1} increased by 53.3% in Black skin, but decreased by 40% in the Asian specimen relative to the White one. Bands in this region may originate from the C=C stretching of conjugated cyclic amines as in pyrroles, which are present in melanin. The C=O stretching vibrations also give rise to IR bands in this region, whether this group belongs to the acetamido (CH_3CONH-) moieties as in glycosaminoglycans (GAG) or to proteins (Amide I). Another significant difference was detected in the band centered at 1565 cm^{-1}, where the spectra of Black skin displayed a 62.5% increment in band intensity, while the Asian specimen showed a 24.7% decrease relative White skin. This band originates from the C-N and C-N-H stretching modes of conjugated cyclic amines as in melanin, the Amide II of proteins, and the acetamido moieties of GAG in their carboxylic acid forms. The salts of GAG, which should predominate *in vivo*, do not absorb in this region. In Black skin, the band centered at 1470 cm^{-1} displayed a 51.1% increment in intensity, but in the Asian specimen, it showed no significant change relative to the White skin. This band is produced, among others, by the C=C stretching vibrations of aromatic groups, which are present in melanin and may be prominent in proteins but are absent in GAG. The data show that relative to White skin, the concentration of C=O groups is 53.3% higher in Black skin and 40% lower in the Asian specimen. Melanin and/or protein (amide II) is 62.5% higher in Black skin and 24.7% lower in Asian skin as compared to the White one. Conjugated cyclic amines and/or aromatic amino acids are 51.1% higher in Black skin than in White skin, but they are not significantly different in the Asian specimen. These results suggest that the concentrations of aromatic amino acids in the proteins of Asian, Black and White skins are significantly different. These data also indicate that FT-IR spectroscopy is useful to characterize normal skin, and to diagnose tissue disorders involving molecules that absorb IR light.

(432) **Specific Induction of Macrophage Inflammatory Protein 1-Alpha in the Brain Regions of Sandhoff Disease Model Mice**

Daisuke Tsuji[1,2], Aya Kuroki[1], Yasuhiro Ishibashi[1] and Kohji Itoh[1,2]
[1] Department of Medicinal Biotechnology, Institute of Medicinal Resources, Graduate School of Pharmaceutical Sciences, The University of Tokushima, Tokushima 770-8505, Japan, [2] CREST, JST, 4-1-8 Honcho, Kawaguchi, Saitama, Japan.

Sandhoff disease is a lysosomal storage disease caused by the simultaneous deficiency of β-hexosaminidase A (HexA; αβ) and B (HexB; β β) due to the primary defect of β-subunit gene (*HEXB*) associated with excessive accumulation of GM2 ganglioside and oligosaccharides carrying the N-acetylhexosamine residues at their non-reducing termini and with neurosomatic manifestations. However, the pathogenic mechanism has not been fully elucidated yet.ÅyPurposeÅzTo examine the neuroinflammatory mechanisms involving in the pathogenesis, we analyzed the expression of chemokines in a Sandhoff disease model mice (SD mice) produced by disruption of the murine Hex β-subunit gene allele (*hexb-/-*). ÅyMethods and ResultsÅzIn RT-PCR analysis, we demonstrated that a chemokine macrophage inflammatory protein-1α (MIP-1α) was induced specifically in the brain regions of SD mice at the early stage of the pathogenesis but not in other systemic organs. On the other hand, little change of the other chemokine mRNAs including RANTES, MCP-1, SLC, Fractalkine and SDF-1 were observed. Quantitative RT-PCR and ELISA experiments also revealed that synthesis of MIP-1α mRNA and the protein in the brain regions, including brain stem and cerebellum, was significantly up-regulated in parallel to the accumulation of natural substrates of HexA and HexB. Immunohistochemical analysis revealed that the MIP-1α immunoreactivity (IR) in the brain regions of SD mice localized in the astrocytes exhibiting glial fibrillary acidic protein (GFAP) IR and/or microglial cells showing Iba1 IR, although the MIP-1α IR was prominent in astrocytes at the late stage of pathogenesis. Immunostaining with anti-GM2 ganglioside and anti-N-acetylglucosaminyl (GlcNAc-) oligosaccharides monoclonal antibodies demonstrated that the latter IR was also found to markedly distribute in microglia and astrocytes in contrast to the neuronal localization of the former IR during pathogenic process.ÅyDiscussionÅzThese results suggest that specific induction of MIP-1α in the microglia and astrocytes resident in the brain regions of SD mice accompanied by accumulation of the GlcNAc-oligosaccharides due to the HexB deficiency might facilitate the activation of microglia and astrocytes leading to the neuroinflammation and neurodegeneration in Sandhoff disease.

(433) **Effects of Leflunomide on Hyaluronan Synthases: Nuclear-Factor-kappa-B independent Suppression of Interleukin-1 induced HAS1 Transcription by Leflunomide**

Karl M. Stuhlmeier.
Kurbadstrafle 10, Vienna, Austria Kurbadstrafle 10, Vienna, Austria.

Hyaluronic acid (HA) is essential in many physiological mechanisms. Nevertheless, unfettered HA release is also associated with diseases such as rheumatoid arthritis. Hyaluronan synovial effusions e.g. serve as a sensitive indicator of altered connective tissue cell function, associated with inflammation. Elevated HA levels in serum can also serve as marker for rheumatoid arthritis (RA) as HA levels in RA patients are elevated to a degree that these can be readily detected. Many reports also demonstrate innumerable pro-inflammatory effects of hyaluronan fragments resulting from unregulated HA release. We hypothesized that uncontrolled hyaluronan release might contribute to genesis and progression of rheumatoid arthritis. Recently published data demonstrating that HA injection does indeed induce RA supports such a concept. We demonstrated earlier that fibroblast-like synoviocytes (FLS) release large amounts of HA in response to most pro-inflammatory cytokines. We further demonstrated that FLS respond to pro-inflammatory cytokines with the activation of HAS1, while HAS2 and HAS3 are mostly unaffected. Here we investigate effects and mechanisms of leflunomide on HAS genes. Leflunomide is a powerful immunomodulating drug that is approved for and successfully used in the treatment of RA. FLS, isolated from RA patients, were treated with 5 and 50 μM leflunomide respectively followed by exposure to IL-1alpha, IL-1beta and PMA for 6 to 24 hours. HA specific ELISA were used to assess HA levels in culture supernatant. HA-synthase assays were utilized to measure leflunomide effects on HA-synthase activity. RT-PCR was utilized to measure mRNA levels of the three HA genes and electrophoretic-mobility-shift-assays (EMSA) were used to test leflunomide's effects on the transcription factor NF-kB. Treatment with leflunomide significantly decreased IL-1alpha, IL-1beta and PMA induced HA levels in culture supernatant in a dose dependent manner. The observed reduction in HA levels is due to inhibition of HA-synthase activity, since HA-synthase assays revealed a dose dependent inhibition by leflunomide. Further experiments, stimulating FLS with IL-1beta or PMA, demonstrated the gene specificity of this drug in that only mRNA levels of HAS1 were affected. Leflunomide has been shown to inhibit the activation and translocation of NF-kB, a transcription factor of utmost importance in the activation of most pro-inflammatory genes. Our data demonstrate that IL-1beta is a powerful activator of NF-kB in FLS. Nevertheless, leflunomide at concentrations that completely blocked IL-1 induced HAS1 mRNA transcription had absolutely no effect on the activation of NF-kB, excluding this well recognized mechanism as the mode of action of leflunomide on IL-1 induced HAS1 activation. Furthermore, restoring the known suppressive effect of leflunomide on pyrimidine syntheses using exogenous uridine was also without effect. Leflunomide was ascribed kinase inhibitor activity as well. We therefore tested a series of kinase-inhibitors and found that specific tyrosine kinase inhibitors can block IL-1beta induced HAS1 mRNA transcription - an effect mimicked by leflunomide. These data indicate the importance of tyrosine kinases in the activation pathway of IL-1beta induced HAS1 activation and might point at novel ways of preventing undesired HAS1 activation. In addition, these findings might also explain some of the drug effects in the treatment of RA.

(434) Control of Chondrocyte Differentiation by Exogenous Proteoglycan

Shusa Ohshika[1], Atsushi Kon[1], Ryoko Uesato[1], Yasuyuki Ishibashi[2], Tomomi Kusumi[3], Satoshi Toh[2] and Keiichi Takagaki[1]

[1] Department of Biochemistry, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan, [2] Department of Orthopaedic Surgery, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan, [3] Department of Pathology, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan.

[Introduction] Autologous transplantation of articular chondrocytes using tissue engineering for treating articular cartilage defects has undergone rapid development. In prolonged monolayer culture, chondrocytes tend to lose gene/protein expressions of articular cartilage-specific extracellular matrices (ECM) such as type II collagen and aggrecan, and morphologically change into fibroblast-like cells, suggesting dedifferentiation. In recent years, various synthetic and natural high polymers have been exploited as scaffolds for maintaining the phenotype of chondrocytes transplanted to articular cartilage defects. However, none of these have been fully satisfactory, therefore, it is necessary to exploit a useful scaffold that maximally exhibits chondrocyte ability. Recently, several studies have demonstrated that proteoglycans (PG), a major component of ECM, may control cell proliferation, differentiation and morphogenesis. In this study, we examined the effect of exogenous PG on proliferation and phenotype expression of cultured chondrocytes embedded in collagen gel. [Materials and Methods] 1. Chondrocyte cultures: PGs prepared from salmon nasal cartilage were mixed with atelocollagen gel and chondrocytes isolated from knee joints of rabbits were embedded in the PG-collagen mixture. As a control group, chondrocytes were embedded in a collagen-medium mixture. 2. Cell numbers: After various period of culturing, chondrocytes in the collagen gel mixture were released by incubation with collagenase. The recovered cells were counted in a hemocytometer. 3. Histologic and immunohistochemical analysis: After 3 weeks of culturing, chondrocyte-containing gels were stained with hematoxylin eosin and alcian blue. The type II collagen was immunohistochemically stained. 4. RT-PCR: Total RNA was extracted from chondrocytes in the collagen gel mixture. The RNA sample was converted to first strand cDNA and PCR was performed. The mRNA expressions of type II collagen, aggrecan and GAPDH were quantified by RT-PCR. [Results] After two weeks of culturing, viable chondrocytes embedded in the PG-collagen mixture were proliferated about one and a half times as many as cells in the control group. Histologically, in both groups, spherical cells which form lacunae and fibroblast-like cells suggesting dedifferentiation were mixed. In the control group, many more fibroblast-like cells were observed than spherical cells. In the PG-collagen mixture, cell morphology was more spherically maintained compared to the control group, and type II collagen and glycosaminoglycan, which are cartilage-specific extracellular matrices, were abundantly synthesized around spherical cells. After eight weeks of culturing, in both groups, gene expression of type II collagen and aggrecan was also maintained, and was slightly greater in the PG-collagen mixture. [Discussion] In this study, exogenous PG not only enhanced chondrocyte proliferation but also histologically formed excellent cartilage-like tissue. It is unknown why PG is effective on cultured chondrocytes embedded in collagen gel. We think that, by mixing of PGs in collagen gel, a microenvironment is created which resembles native cartilage, and modulates the bioactivity of chondrocytes. Another reason for differences in the cellular response between the groups might be the availability of growth factors. Here, we demonstrated for the first time the effect of exogenous PG on three-dimensional cultured chondrocytes. The results suggest that exogenous PG may be useful as a material for cartilage regeneration.

(435) A Novel Inhibition Mechanism of Hyaluronan Synthesis by 4-Methylumbelliferone

Ikuko Kakizaki[1], Kaoru Kojima[1], Keiichi Takagaki[1], Masahiko Endo[1], Reiji Kannagi[2,4], Tadashi Yasuda[3], Satoka Mita[3,5], Koji Kimata[3] and Naoki Itano[3,4]

[1] Department of Biochemistry, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan, [2] Program of Molecular Pathology, Aichi Cancer Center, Research Institute, Nagoya 464-8681, Japan, [3] Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-1195, Japan, [4] Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Japan, [5] Department of Biochemistry, Osaka University Medical School, Suita, Osaka 565-0871, Japan.

Introduction There are a considerable number of reports showing that the elevation of hyaluronan biosynthesis is associated with tumorigenesis and cancer progression. Specific inhibitors of hyaluronan biosynthesis would therefore serve as valuable therapeutic agents to prevent the malignant alteration of cancer. 4-methylumbelliferone (MU) has been shown to inhibit hyaluronan synthesis in human skin fibroblasts and in group C *Streptococcus*. Here, we investigated the inhibition mechanism of MU in rat 3Y1 fibroblasts stably expressing mouse hyaluronan synthase 2 (HAS2). **Methods & Results** The HAS2 transfectants were cultured in the presence of various concentrations of MU. Pericellular hyaluronan matrices surrounding the cells were visualized by the particle exclusion assay and hyaluronan contents in the conditioned medium were measured by ELISA-like assay. MU reduced hyaluronan synthesis and matrix formation of the HAS2 transfectants in a dose-dependent manner. Transcription of the exogenous HAS2 and endogenous HAS genes in the transfectants was then assessed by real-time quantitative RT-PCR. The evaluation of HAS transcripts and analysis of cell-free hyaluronan synthesis suggested the posttranscriptional suppression of HAS activity by MU. The posttranscriptional suppression of HAS activity was also observed using *p*-nitrophenol, a well known substrate for UDP-glucuronyltransferases (UGTs). We examined whether the inhibition was exerted by glucuronidation of MU by HPLC, TLC, and Mass spectrometric analyses. The level of MU glucuronidation was well correlate with the inhibition level of hyaluronan synthesis although MU-glucuronide (MU-GlcUA) did not directly inhibit hyaluronan synthesis. To clarify the role of UGT in the MU-mediated inhibition of hyaluronan synthesis,

recombinant UGT1A isoforms were exogenously added into the cell-free hyaluronan synthesis. The increased MU glucuronidation by UGT enhanced the inhibition of hyaluronan synthesis by MU. Similar enhancement of the inhibition was also observed when human UGT1A6 was overexpressed into COS cells. Conversely, the inhibition by MU was rescued by adding increased concentrations of UDP-GlcUA into the cell-free hyaluronan synthesis system. **Conclusion** We propose a novel mechanism for the MU-mediated inhibition of hyaluronan synthesis involving the glucuronidation of MU by endogenous UGT other than HAS. The information presented here will be useful in the development of new inhibitors targeting hyaluronan biosynthesis with improved efficacy.

Reference, Kakizaki I, *et al. J. Biol. Chem.* 279, 33281-33289, 2004.

(436) **A Crucial Role of Plasma Membrane-Associated Sialidase (NEU3) in the Survival of Human Cancer Cells**

Taeko Miyagi, Tadashi Wada, Setsuko Moriya, Seiji Ueno, Kengo Kato and Kazunori Yamaguchi

Division of Biochemistry, Miyagi Cancer Center Research Institute, and CREST, JST, Japan.

Human plasma membrane-associated sialidase (NEU3), which specifically hydrolyzes gangliosides, has been implicated in cellular events including neuronal differentiation (1), transmenbrane signaling (2), and carcinogenesis (3). We previously demonstrated a marked increase in NEU3 expression in various human cancers including colon (4) and prostate cancers. The mRNA and the activity levels were increased in cancer tissues compared to adjacent non-tumor tissues. In situ hybridization exhibited sialidase expression in epithelial elements of adenocarcinomas. To understand the significance of the increased expression, NEU3 changes in the cultured human cancer cells were examined during differentiation and apoptosis induced by sodium butyrate treatment.The sialidase level was down-regulated, when lysosomal sialidase (NEU1) was up-regulated, suggesting that up-regulation of NEU3 leads to suppression of cell differentiation and apoptosis. Transfection of the *NEU3* gene into cancer cells, in fact, inhibited apoptosis accompanied by increased Bcl-2 and decreased caspase expression. To elucidate further the molecular mechanism and the physiological function of NEU3, we have employed small interference RNA (siRNA) approaches in DLD-1 cells and HeLa cells. Transfection of siRNA for *NEU3* into these cells induced apoptosis, as assessed by annexin V detection and MTT assay, but the scrambled control nucleotides did not cause apoptosis, when the mRNA level was reduced by 70%-90% with siRNA as compared with the scrambled control nucleotides. In DNA micro array with HeLa cells the siRNA transfection was accompanied by increased expression of apoptosis -associated molecules such as TNF receptor, TRAIL receptor,caspase -7 and -9, and also by increase in cell differentiation-related proteins including melanoma differentiation associated gene-7. In contrast, overexpression of *NEU3* brought about decreased expression of these molecules and increased Ras family, resulting in suppression of apoptosis as expected in the previous experiments. In summary, knock down of *NEU3* gene induced cell apoptosis and overexpression caused its inhibition, indicating that *NEU3* represents an essential gene for cell survival and the marked increase in cancer cells accelerates their insusceptibility to apoptosis. Although the direct target molecule for NEU3 remains to be determined, the siRNA could have potential utility for the gene-based therapy of various human cancers.

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(437) **Identification of Sialo-Sugar Receptors for Influenza Viruses**

Masami Okumura[1,2,3], Kazuya IPJ Hidari[1,2,3], Rieko Mizuno[1], Shotaro Iwamoto[1,2,3], Kazuo Kon[5], Susumu Ando[5], Yoshiki Yamaguchi[2,6], Koichi Kato[2,6], Tadanobu Takahashi[1,2,3], Chao-Tan Guo[1,2,3,4], Daisei Miyamoto[1,2,3], Takashi Suzuki[1,2,3] and Yasuo Suzuki[1,2,3]

[1] Department of Biochemistry, University of Shizuoka, School of Pharmaceutical Sciences, [2] JST, CREST, [3] COE Program in the 21st Century, [4] Zhejiang Academy of Medical Science, China, [5] Tokyo Metropolitan Institute of Gerontology, [6] Department of Structural Biology and Biomolecular Engineering, Graduate School of Pharmaceutical Sciences, Nagoya City University.

Receptor molecules specific for influenza viruses are functionally expressed on the host cell surface. Entry of viruses into the cells is mediated by the interaction between the sialo-sugar receptors and influenza virus hemagglutinin. In this study, we identified functional endogenous receptor glycolipids responsible for influenza virus infection. We isolated the acidic glycolipids bound to influenza viruses from chick allantoic membrane. Fractions containing these glycolipids clearly showed the inhibition of influenza virus infection to MDCK cells. A purified mono-sialylated glycolipid was strongly bound to influenza A viruses which have preferentially recognize terminal Neu5Ac alpha 2-3 residue. Since relative content of this glycolipid is the highest in all acidic glycolipids bound to influenza viruses, this molecule may be the main receptor for certain types of influenza viruses, for instance, avian influenza viruses. Mass spectrometry and 1H-NMR analyses demonstrated that the carbohydrate structure of the glycolipid is the same as that of authentic alpha 2-3 sialylparagloboside. Di-sialyl glycolipids were also strongly bound to both A and B types of influenza viruses we tested. These molecules possibly have branched type of carbohydrate chains with different linkage of terminal Neu5Ac residues, such as Neu5Ac alpha 2-3 Gal and Neu5Ac alpha 2-6 Gal. Therefore, these glycolipids we isolated may be new functional receptor molecules for influenza viruses.

(438) **Saposin D Deficiency Causes Accumulation of Hydroxy Fatty Acid-Containing Ceramides in the Kidney and Brain with Renal Tubular Cell and Cerebellar Purkinje Cell Degeneration in the Mouse**

Keiko Tadano-Aritomi[1], Junko Matsuda[2], Makiko Kido[2], Kumiko Tominaga[2], Yasuhiro Kuroda[2], Kunihiko Suzuki[3,4] and Ineo Ishizuka[1]

[1] Department of Biochemistry, Teikyo University School of Medicine, [2] Department of Pediatrics, University of Tokushima Graduate School of Medicine, [3] Institute of Glycotechnology, Tokai University, [4] Departments of Neurology and Psychiatry, University of North Carolina.

The sphingolipid activator proteins (saposins A, B, C, D) are small homologous glycoproteins that are encoded by a single gene within a large precursor protein (prosaposin). They are specifically required for *in vivo* degradation of sphingolipids with short carbohydrate chains. Human patients of the prosaposin deficiency and specific saposin B or C deficiencies are known. In addition, mouse models of prosaposin deficiency and saposin A deficiency have been generated. Saposin A-/- mice exhibited the phenotype of a chronic form of globoid cell leukodystrophy, confirming that saposin A is indispensable for degradation of galactosylceramide. There are reports suggesting that saposin D is an acid ceramidase activator, but no specific saposin D deficiency is known so far. Recently, a mouse model was generated by introducing a mutation in the saposin D domain of the prosaposin gene. Saposin D-/- mice developed progressive polyuria at around 2 months and ataxia at around 4 months. The kidney showed renal tubular degeneration and eventual hydronephrosis. In the nervous system, progressive loss of cerebellar Purkinje cells was conspicuous, and almost all Purkinje cells disappeared by 12 months.

In view of the suggested function of saposin D as an activator for *in vivo* degradation of ceramide, we analyzed the brain, kidney and liver tissues from homozygous mutant mice. By TLC of the total lipid extract, an alkaline-resistant lipid, which moves more slowly than ceramides containing nonhydroxy fatty acids and 4-sphingenine (NFA/d18:1), was detected in the kidney and brain of saposin D-/- mice. By liquid secondary ion and electrospray ionization mass spectrometry, this lipid was identified as a ceramide containing hydroxyl fatty acid (HFA/d18:1), which was barely detectable in the wild type mice. In the kidney, other molecular species of ceramide which consist of HFA and 4-hydroxysphinganine (HFA/t18:0) were also increased. NFA-ceramides (NFA/d18:1), which accumulate in the tissue of human Farber disease, also increased in saposin D-/- mice but to a lesser extent. The accumulation of HFA-ceramides (HFA/d18:1) was already evident at 40-day in the kidney. In the brain, the accumulation was first detected around 6-7 months and more prominent in the cerebellum than in the cerebrum. Consistent with the absence of histopathological changes, there was no accumulation of HFA-ceramide in the liver. No major abnormalities in the profile of other lipids including sulfatides and gangliosides were found in saposin D-/-, except for dihexosylceramide, which increased 2-3 times in the kidney. These data confirmed that saposin D is an activator for ceramide degradation by acid ceramidase *in vivo*. Specific accumulation of HFA-ceramides in saposin D-/- mice may suggest that saposin D has more affinity with HFA-ceramides than NFA-ceramides and is more indispensable for degradation of HFA-ceramides than NFA-ceramides. These results not only indicate the role of saposin D in *in vivo* ceramide metabolism but also suggest possible cytotoxicity of HFA-ceramide underlying the cerebellar Purkinje cell and renal tubular cell degeneration.

Reference:Å@Matsuda J, *et al., Hum. Mol. Genet.*, provisionally accepted, August 3, 2004

(439) **Carbohydrate Alterations on Cell Membrane of Immunogenic and Non-immunogenic Tumor Lines**

Smadar Tal[1], Shachar Dotan[2], Yaccov Kerlin[2], Ron Apte[2] and Rachel Glicklis[1]

[1] The National Institute of Biotechnology and The Department of Biotechnology Engineering Ben-Gurion University, Beer-Sheva, Israel, [2] The Department of Microbiology and Immunology Ben-Gurion University, Beer-Sheva, Israel.

It has been demonstrated that malignant transformation is associated with alterations in the glycosylation process. We now demonstrate cancer-associated alterations in membrane carbohydrate structures of immunogenic vs. non-immunogenic tumor cell lines. The immunogenic 3-MC-induced fibrosarcoma cell lines, arising in IL-1 alpha deficient mice, were failed to develop into tumors in secondary recipients. In contrast, non-immunogenic 3-MC-induced fibrosarcoma cells, arising in control BALB/c mice, developed invasive tumor. In a model of sub-lethally irradiated mice however, the immunogenic cells from IL-1 alpha deficient mice were able to develop tumors, indicating that in normal recipients the malignant cells are eradicated by the immune system, which some components of it are missing in the IL-1 alpha deficient mice. To elucidate the origin of these phenomena, we measured the number of cells that are capable of binding carbohydrate specific lectins, such as SNA, Concavalin A (Con A), PNA, Jacalin, PNA and WGA using FACS flow cytometry. The results with SNA showed that the non-immunogenic fibrosarcoma cell lines contain more sialic acid. PNA binding to beta-Gal, GalNAc was inhibited by sialic acid in the non-immunogenic cell lines. Con A FACS analysis showed that binding to mannose sites is similar for both cell lines however, on per cell basis, the immunogenic cell lines showed higher intensity. This may indicate that more mannose or hybrid structures appear in this type of line. Jacalin, which has similar functionality as PNA but is not affected by sialic acid presence, was found to bind both lines to the same extent. As well, results performed with WGA, which binds GlcNAc and GlcNAc chains showed similar binding. The described results were repetitive for two lines originated from the immunogenic fibrosarcoma, alpha 20, M3, as well as for the non-immunogenic fibrosarcoma, BALB/c 25 and 29. We then tried to relate these carbohydrate alterations with membrane-protein using western blot with Con A, SNA, Jacalin and WGA. The western results with SNA showed more glycoprotein bands in the non-immunogenic cell lines. The other blots didn't show any significant differences between the lines. Taken together, our results indicate that tumor immunogenicity may be associated with alterations of carbohydrates on cell membrane. Our future goals are to characterize by mass spectrometry the altered carbohydrate structures as appear on membrane glycoproteins of the pairs of lines and to relate these alterations with the type of the membrane glycoproteins.

(440) **Recombinant GNE Alters the Expression of Proteins Important for Muscle Functions and Provides Insights into the Cellular Basis of HIBM**

Hao Chen[1], Stephan Hinderlich[2] and Kevin J Yarema[1]

[1] Department of Biomedical Engineering,Johns Hopkins University,Baltimore MD 21218,USA, [2] Charite-Universitatsmedizin Berlin, Campus Benjamin Franklin,Institut fur Biochemie und Molekularbiologie,Arnimallee 22,14195 Berlin-Dahlem,Germany.

UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase (GNE) is a key enzyme in cellular sialic acid production; it catalyzes the first two steps of sialic acid biosynthesis in the cytosol. Recent studies have shown that inactivation of GNE by gene targeting causes early embryonic lethality in mice; this finding emphasizes the fundamental role of this bifunctional enzyme and sialylation during development. Abnormalities associated with GNE can also be manifest much later in life, as demonstrated by the human disease hereditary inclusion body myopathy (HIBM). Genetic evidence has linked single amino acid mutations distributed throughout both catalytic domains of GNE with this unique group of neuromuscular disorders characterized by adult onset,slowly progressive weakness,and typical muscle pathology. The most obvious molecular basis of HIBM, a lack of cellular sialic acid production due to reduced GNE activity observed by in vitro tests, does not appear to apply to human patients as several studies have shown they have close to normal levels of sialic acid. We are exploring two sets of experiments to determine if GNE plays a non-sialic acid-related role in GNE. First, because the substrate of GNE, UDP-GlcNAc, is shared with OGT we are investigating the effects of mutant GNE on both OGT expression and the resulting O-GlcNAc protein modification. Second, we transient transfected GNE gene into the mouse muscle cell (C2C12) lines and determined the differential gene expression using cDNA microarray analysis by/with Affymetrix Mouse Genome 430 2.0 Array. These results showed that 27 of genes were highly up-regulated by over-expressed GNE, 50 of genes were down-regulated. The level of expression of selected genes was independently confirmed by real time RT-PCR. Notable genes undergoing changes include the holocytochrome c-type synthetase (HTSC), α-galactosidase, hypothalamus that was up-regulated to 5-fold, 10-fold, and 2-fold, respectively. Conversely, Dystroglycan (DG), serine (or cysteine) proteinase inhibitor (PI-A), ADP-ribosylation factor 6 (ADPRF6), protein tyrosine phosphatase S-receptor (PTPPRS) were down-regulated to 5-fold, 15-fold, 5.5-fold, and 2-fold. Considering that several of the genes that displayed significant changes in expression are responsible for the functions of structural proteins, enzyme, transport, molecular dynamics and nucleic acid binding in muscle cells; these results may provide a clue to the understanding of the HIBM. Accordingly, we are testing whether mutant forms of GNE that occur in HIBM have similar or different effects on the expression of muscle-specific proteins.

(441) **Feedback Control Mechanisms, not the Potential Catalytic Epimerization Activity of GNE, Determines Bulk Metabolic Flux through the Sialic Acid Biosynthetic Pathway**

Zhiyun Wang[1], Zhonghui Sun[1], Hao Chen[1], Stephan Hinderlich[2] and Kevin J Yarema[1]

[1] Whitaker Biomedical Engineering Institute, The Johns Hopkins University, 3400 N.CharlesSt, Baltimore MD 21218 , [2] Charite - Universitatsmedizin Berlin, Campus Benjamin Franklin, Institut fur Biochemie und Molekularbiologie, Arnimallee 22, 14195 Berlin-Dahlem, Germany.

Cell surface display of sialic acid is of great interest due to the many critical functions known to be governed by this sugar. The UDP-GlcNAc 2-epimerase/ManNAc 6-kinase (GNE) bifunctional enzyme is the key regulator of flux into the sialic acid biosynthetic pathway and, by extension, exerts influence over cell surface display of this sugar. Many aspects of sialylation, however, remain inadequately resolved. For instance, single amino acid GNE mutations found in the disease hereditary inclusion body myopathy (HIBM) reduce the activity of this enzyme by as much as 95% when tested by using in vitro assays. By contrast, sialic acid levels in living cells or tissue derived from HIBM patients harboring GNE mutations often remains close to normal. We sought to resolve this apparent discrepancy by creating a panel of recombinant GNE mutants that allow the effects of enzyme activity and feedback control to be assessed both independently and jointly. To explain, HIBM mutations occur in the catalytic domains of GNE and impact either the catalytic epimerase or kinase activity of this protein. Another set of mutations found in the disease sialuria occur in the regulatory domain of GNE and alleviate allosteric feedback inhibition of epimerase activity that normally takes place upon CMP-Neu5Ac (a downstream metabolite) binding. We expressed recombinant forms of GNE with sialuria and/or HIBM mutations in human HEK293 cells to test the relative importance of feedback control regulation and the potential catalytic activity of GNE. Expression of the R266Q °∞sialuria°± form of GNE increased total cellular levels of sialic acid by ~10 fold; this response was expected because of the defective feedback inhibition that results from this mutation. When the R266Q mutation was combined with the R177C, D225N, R246Q and R306Q HIBM mutations in the same gene sequence, the double mutants resulted in a similar ~10-fold elevation in cellular sialic acid. This result was unexpected considering that HIBM mutations decrease GNE epimerase activity in vitro and consequently would not be expected to support increased sialic acid production to the same extent as GNE with wild-type catalytic domains. This finding demonstrated that cellular levels of sialic acid are determined primarily by feedback control mechanisms rather than by the potential upper limit of the catalytic activity of GNE. This work provide a theoretical basis to explain discrepancies between clinical observations where sialic acid levels are often-times close to normal in HIBM patients whereas in vitro tests of the HIBM forms of GNE show various degrees of reduced activity. Further, the analysis of the disconnect between GNE levels in cells ®C this protein is maintained at much higher levels than necessary to supply the sialic acid pathway as evidenced by the need for the feedback inhibition mechanism that limits actual epimerase activity to a small fraction of its potential activity ®C implies that GNE may have a second cellular function and it is the impairment of this non-

sialic acid metabolism-related activity that contributes to disease symptoms of HIBM.

(442) **The Interaction of Galectin-1 with Mast Cells Promotes Surface Exposure of Phosphatidylserine without Inducing Apoptosis or Degranulation.**

Daniel R Callejon[1], Marlise AB Montes[1], Lilian Cataldi[1], Rodrigo Orlandini-Castro[2], Maria C Jamur[2], Constance Oliver[2], Sean R Stowell[3], Richard D Cummings[3] and Marcelo Dias-Baruffi[1]

[1] Faculdade de Ciencias Farmaceuticas de Ribeirao Preto (DACTB) - Universidade de Sao Paulo - Brasil, [2] Dept of Molecular Biology and Cell Biology - Faculty of Medicine of Ribeirao Preto - University of Sao Paulo - Brazil, [3] Dept of Biochemistry and Molecular Biology - University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104.

Galectin-1 (Gal-1) is involved in several biological processes, including modulation of the inflammatory response. Mast cells play a critical role in inflammatory events, however, little is known about the impact of Gal-1 on mast cell biology. In this study, we investigated the ability of Gal-1 to promote morphological changes, apoptosis and degranulation in mast cells. The induction of morphological changes on the surface of a mast cell line (RBL-2H3) was evaluated by phase, scanning electron, and fluorescence microscopy. The exposure of phosphatidylserine (PS) and induction of DNA degradation were determined by a flow cytometry using annexin V-FITC and the Tunel reaction, respectively. Cell degranulation and cell viability were assessed by monitoring the release of the secretory granule enzyme β-hexosaminidase (β-HEX) and by the MTT/formazan-assay, respectively. After treatment with Gal-1 (10-20 microMolar), the shape of the RBL-2H3 cells changed and this was accompanied by changes in the distribution of F-actin as assessed by staining with Texas Red-Phalloidin. Gal-1 (20 microMolar) also induce ruffling of the plasma membrane. RBL-2H3 treated during 4 hours with Gal-1 (20 microMolar) showned staining of RBL2H3 cells with with Annexin V-FITC (65±2.1%). However, RBL-2H3 cells stimulated with Gal-1 (20 microMolar) did not shown DNA fragmentation by Tunel assay (7.9±1.3%), whereas RBL-2H3 cells plus DNAse I (30U/mL) showned 65.4±2.1% DNA fragmentation. Also, RBL-2H3 cells treated with Gal-1 (2.5 to 40 microMolar) remained viable, as demonstrated by the high level of formazan production by these cells, similar to untreated cells. The percentage of β-HEX release induced by 20 microMolar of Gal-1 (3.3±0.3%) was similar to the negative control (1.2±0.4%). In contrast, the release of β-HEX induced by stimulation of these cells through FceRI (IgE anti-DNP + DNP-HSA) was 58 ±2%. Interestingly, Gal-1 (20 microMolar) promotes a partial inhibition (31±2%) of degranulation induced by FceRI stimulation. These results suggest that interaction between Gal-1 and mast cells promotes cell activation and exposure of PS without inducing apoptosis or degranulation. In addition, these data may contribute to further studies on mast cell homeostasis and role of Gal-1 in inflammatory diseases associated with mast cells. Financial Support: FAPESP - NIH

(443) **Detection of Galectin-1 and Anti-galectin-1 Antibodies in Serum and Cerebrospinal Fluid derived from Lupus Patients.**

Renata Delalibera-Joviliano[1], Juliana S Oliveira[1], Marlise AB Montes[1], JosÈ C Rosa[2], Lewis J Greene[2], Eduardo A Donadi[3], Richard D Cummings[4] and Marcelo Dias-Baruffi[1]

[1] Faculdade de Ciencias Farmaceuticas de Ribeirao Preto (DACTB) - Universidade de Sao Paulo-Brasil, [2] Dept of Molecular Biology and Cell Biology and Protein Chemistry Center - Faculty of Medicine of Ribeirao Preto - University of Sao Paulo - Brazil, [3] Faculty of Medicine of Ribeirao Preto - University of Sao Paulo - Brazil, [4] Depto of Biochemistry and Molecular Biology - University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104.

Galectin-1 (Gal-1) is a 14 kDa protein that belongs to a lectin family that recognizes β-galactosides. This lectin participates in several biological processes, including modulation of inflammation and axonal regeneration. In this study we evaluated the presence of Gal-1 (native and inative forms) and anti-Gal-1 (Ab-Gal-1) antibodies levels in both serum and cerebrospinal fluid (CSF) of neuropsychiatric lupus (NPL) patients and patients presenting other clinical manifestations, including cutaneous or renal lupus. We studied 21 women with active NPL and 21 healthy women matched the NPL group by age. The determination of components were evaluated by ELISA and Western-blotting using a monoclonal antibody anti-human Gal-1. To detect the active Gal-1 forms in the samples, we used the laminin-binding assay and affinity chromatography on Lactosyl-Sepharose. The bound material was submitted to SDS-PAGE and a 14 kDa band was submitted to in situ trypsin gel digestion and the tryptic peptides were used to identify the protein by mass spectrometry. Recombinant human galectin-1 was used as an internal reference on all experiments. Compared to the control group and to the other clinical manifestations of lupus, a 50% increase of serum and CSF levels of Gal-1 and Ab-Gal-1 ($p < 0.001$) was only verified in samples from the NPL group. The mass fingerprint analysis of trypitic peptides of a 14 kDa protein recovered from the NPL samples unequivocally identified this protein as Gal-1 and not other related galectin. In conclusion these preliminary data suggest that active Gal-1 which is present in NPL patients could be involved with the ethiopathology of NPL and that this lectin may have a protective role in this autoimmune disease. Financial Support: FAPESP - NIH

(444) **A New Case of Congenital Disorder of Glycosylation Type II.**

Erik A Eklund, Chui Sien Chan, Yoshi Miura and Hudson H Freeze

Program for Glycobiology and Carbohydrate Chemistry, The Burnham Institute, La Jolla, CA 92037.

Type II Congenital disorders of glycosylation (CDG) are caused by defects in the biosynthesis or processing of protein-bound oligosaccharides. Here we describe a child with marked developmental delay, seizures, hypotonia and decreased reflexes, consistent with known types of CDG. Electrospray ionization mass spectrometry of his transferrin revealed the specific loss of one sialic acid residue from oligosaccharides on at least half of the transferrin molecules. Furthermore, separation of PNGaseF-released/2-aminobenzamide labeled oligosaccharides from total serum glycoproteins, based on charge, showed a decrease in chains with two and more sialic acids. MALDI analysis of these chains revealed an accumulation of complex biantennary chains with only one sialic acid. No effects on O-linked sialylation were seen using [3H]galactose labeling in the presence of α-phenyl-GalNAc (GAP) in patient and control fibroblasts. This indicates that the patient's defect is specific to the N-linked pathway. Since most sialic acids on human serum transferrin oligosaccharides and those of total human serum are α2,6-bound to their penultimate galactose, we wanted to test whether the loss was specific to this epitope or if α2,3-Sia also was decreased on N-linked glycans. Fibroblasts mainly synthesize structures with α2,3-bound Sia and labeling of fibroblasts from the patient and a control with [3H]mannose with subsequent enzymatic release and charge separation of the labeled N-linked chains revealed only minor differences. Furthermore, patient and control fibroblasts were incubated with the α2,6-Sia-specific *Polyporus squamosus* lectin (PSL):FITC and analyzed by FACS. Patient cells showed slightly less binding, suggesting less α2,6-Sia-epitopes on their cell surface. Taken together, the data suggest the specific loss of α2,6-Sia on N-linked oligosaccharides. One single enzyme, ST6Gal-I, is responsible for the formation of this specific epitope. However, sequencing of the ST6Gal-I cDNA from patient fibroblasts did not reveal any mutations. Also, the message level of ST6Gal-I appeared normal in the patient cells. Several plausible explanations for defective activity of a glycosyltransferase without exonic mutations exist. Intronic mutations can affect splicing in specific tissues. ST6Gal-I from rat liver has been shown to have two RNA transcripts differing in only one nucleotide, possibly arising from post-transcriptional editing. Mutations affecting this editing process may be of importance for the ST6Gal-I activity. Another possibility is that ST6Gal-I needs a chaperone for full activity (similar to Core 1 galactosyltransferase) and mutations in such a gene may also be an explanation. Further investigation is under way. (Supported by RO1 DK55615)

(445) **Mismatched Hemagglutinin and Neuraminidase Activities in Recent H3N2 Influenza Viruses**

Upma Gulati[1], Wenxin Wu[1], Shelly Gulati[1], Joseph L. Waner[2] and Gillian M. Air[1]

[1] Department of Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center, [2] Department of Pediatrics, University of Oklahoma Health Sciences Center.

The hemagglutinin (HA) of influenza viruses initiates infection by binding to sialic acid on the cell surface. Human viruses recognize predominantly α2,6 linked sialic acids while avian viruses show preference for the α2,3 linkage. The influenza neuraminidase (NA) cleaves sialic acid, and was originally called 'receptor destroying activity'. The influenza NA can cleave both α2,3 and α2,6 linked sialic acids, but all influenza NAs have higher activity for the α2,3 linkage. Recent H3N2 influenza viruses have lost the ability to bind to chicken red blood cells. This has been found to involve

lower affinity for α2,6 sialic acid, so that although there is a considerable amount of α2,6 sialic acid on chicken red cells, the density is too low for agglutination (Nobusawa et al. Virology 278:587-96, 2000; Mochalova et al. Virology 313:473-80, 2003). We find that many human influenza viruses, including recent isolates passaged only in mammalian cells, have mismatched HA and NA specificities, in that the viral NA does not elute virus from red blood cells. Some viruses elute at room temperature but bind back at 4∞, showing that the 'elution' is due to low affinity rather than to NA activity. Other viruses bind very tightly to red blood cells and elute only after digestion by a highly active, broad specificity, bacterial sialidase. In some vaccine-adapted influenza virus strains, this lack of elution correlates with very low levels of viral NA activity. Recent 'Fujian-like' H3N2 viruses do not elute from red blood cells. The Fujian-like viruses bind to human red cell glycophorin. We have shown that red cell binding is insensitive to the viral NA but is sensitive to Micromonospora viridifaciens sialidase. We conclude that only specific form(s) of sialic acid are used as receptor(s) by recent human H3N2 influenza viruses and the identities of these are under investigation.

(446) **Semi-synthesis of Glycoproteins and Mimetics using Chemoselective Ligations**

Derek Macmillan.
School of Chemistry, University of Edinburgh, Kings Buildings, West Mains Road, Edinburgh, EH9 3JJ, Scotland, UK..

Chemoselective chemical reactions have proven themselves as powerful tools for the assembly of natural and unnatural (but nevertheless highly complex) biopolymers. We have employed expressed protein ligation,[1] which is a selective coupling reaction between bacterially-derived peptide thioester or N-terminal cysteine containing components and synthetic glycopeptide thioester or N-terminal cysteine containing components, to assemble the protein backbone.[2-3] This reaction occurs in aqueous solution, in the absence of protecting groups and ultimately forms a native peptide bond at the site of ligation. Oligosaccharides can be easily introduced as glycosylated amino acids during peptide synthesis or through another chemoselective coupling reaction between alfa-haloacetamide sugars and the thiol groups of cysteine residues, giving rise to multiply and site-specifically glycosylated glycoproteins and glycoprotein mimics.[4] Here we present our progress on the application of such chemoselective coupling reactions to the synthesis of the glycoprotein hormone erythropoietin (EPO) [5] and describe our efforts towards elaborating the oligosaccharide appendages that confer biological activity. It is hoped that the generality of such coupling strategies and the reduced burden of chemical synthesis may expedite the production of defined glycoproteins. → References: [1] T.W. Muir Annu. Rev. Biochem., 2003, 72, 249-289. [2] D. Macmillan and C. R. Bertozzi, Tetrahedron, 2000, 56(48), 9515-9525. [3] D. Macmillan and C. R. Bertozzi, Angew. Chem. Int. Edn., 2004, 43(11), 1355-1359. [4] D. Macmillan, A.M. Daines, M. Bayrhuber and S.L. FIitsch, Org. Lett., 2002, 4(9), 1467-1470. [5] D. Macmillan and L. Arham, J. Am. Chem. Soc., 2004, 126 (31), 9530-9531.

(447) **Human GM3 Synthase Deficiency: Autosomal Recessive Infantile Onset Symptomatic Epilepsy Syndrome.**

David A. Priestman[1], David C. A. Neville[1], Gabriele Reinkensmeier[1], Michael A. Simpson[2], Christos Proukakis[2], Michael A. Patton[2], Raymond A. Dwek[1], Terry D. Butters[1], Frances M. Platt[1] and Andrew H. Crosby[2]
[1] Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford, OX1 3QU, UK, [2] Department of Medical Genetics, St. George's Hospital Medical School, University of London, Cranmer Terrace, London SW17 0RE, UK.

A deficiency of GM3 synthase in humans results in an infantile onset symptomatic epilepsy syndrome associated with developmental stagnation and blindness. We have studied 8 affected individuals with this syndrome in two families from a large Old Order Amish pedigree. Gene sequencing identified a nonsense mutation in the SIAT9 gene, which is predicted to result in the premature termination of the GM3 synthase enzyme (CMP-NeuAc: lactosylceramide α-2,3 sialyltransferase, EC 2.4.99.9). GM3 synthase is a member of the sialyltransferase family and catalyses the initial step in the biosynthesis of the majority of complex ganglioside species from lactosylceramide (LacCer). Biochemical analysis of plasma glycosphingolipids (GSLs) confirmed a lack of GM3 synthase activity in all the affected individuals. There was a complete absence of GM3 ganglioside and its biosynthetic derivatives, and an increase in LacCer and its alternative neutral GSL derivatives, Gb3 and Gb4. While the relationship between defects in ganglioside catabolism and a range of lysosomal storage diseases is well documented, this is the first proven report of disruption of ganglioside biosynthesis associated with human disease.

(448) **Early Detection of Liver Fibrosis and Cirrhosis using N-Glycan Markers: The Next Generation**

Wouter Laroy[1], Nico Callewaert[1,2], Hans Van Vlierberghe[3] and Roland Contreras[1]
[1] Unit for Fundamental and Applied Molecular Biology, Dept. for Molecular Biomedical Research, Ghent University and Flanders Interuniversity Institute for Biotechnology (VIB), Technologiepark 927, B-9052 Zwijnaarde, Belgium, [2] Present address: Zurich Glycomics Initiative, Swiss Federal Institute of Technology (ETH), Schmelzbergstrasse 7, CH-8092 Zurich, Switzerland, [3] Department of Gastroenterology and Hepatology, Ghent University Hospital, De Pintelaan 185, B-9000 Gent, Belgium.

The amount of functions needed for human live largely exceeds the number of genes found in the human genome. Consequently, one gene must encode different functions. Next to several degrees of transcriptional control, post-translational modifications of the gene products have an important role in the tuning of protein functions. As a result of different or altered environmental factors or physiological conditions, cells synthesize differently glycosylated proteins. In pathology, this has two consequences. First, there may be a connection between the different glycans and the outcome, progress or seriousness of the disease. Based on this knowledge, therapies may be designed. Second, these changes may be used to detect the diseases in early stage. Here, they function as biomarkers.

As for many other diseases, early detection of liver disease is important in order to decide on therapy and to improve healing potential. Gold standard for detection of liver fibrosis and cirrhosis is a biopsy, an invasive technique with high cost and risk. As an alternative, good serological biomarkers are of big interest. Previously, we have reported on an N-glycan serum biomarker (Callewaert et al., 2004). Using classical DSA-FACE, a sensitive glyco-profiling technique, N-glycans in minimal amounts of serum are analyzed after sialidase treatment. High sensitivity and specificity markers for both detection of cirrhosis (79% and 86% respectively; both are 100% for decompensated cirrhosis) and follow-up of fibrosis were characterized. Now, improvements to both the isolation and the analysis of the N-glycans are reported.

To be able to use the glycan marker in clinical laboratories, more straightforward preparation techniques are wanted. Using standard PCR equipment, a technique for the preparation of modified N-glycans (from isolation, over desialylation to fluorescent labeling) from minimal amounts of serum was set up and optimized. Only 96-well plates are used, and the number of transfer steps is kept to an absolute minimum. The resulting glycans can be directly analyzed on slab-gel sequencing platforms without prior cleanup. We show that the old and new method have good linear correlation.

As an alternative for slab-gel systems, capillary sequencing equipment with LIF detection was tested. Next to a higher throughput and a big reduction in analysis time, improved sensitivity and resolution was obtained. This opens new perspectives, not only for the research on liver disease, but also for glycomics research in general.

As for many other biomarkers, inter- and intra-patient variation of prognostic values is significant within patient groups. Currently, we are checking the possible variation of N-glycan profiles in a healthy volunteer group. The use of plasma or serum, the time between whole blood taking and processing, the time of blood taking and the diet of the volunteer are under investigation.

Ref. Callewaert et al. (2004) Nature Medicine. 10, 429-432.

WL is a postdoctoral fellow with DWTC. NC has a honorary fellowship from the Fund for Scientific Research Flanders (FWO). This work was supported by FWO and by Ghent University (GOA Grant 12052299).

(449) **Detailed Dissection of rppGalNAc-T1 glycosylation of Mucin Peptides by NMR Spectroscopy**

Jean Gariepy, Richard Brokx and Leigh Revers
Department of Medical Biophysics, University of Toronto.

MUC1, the prototypic human mucin, is a ubiquitous membrane-associated glycoprotein that is abundant on the apical cell-surfaces of epithelial tissue. The molecule is characterized by an extended extracellular domain comprising a proline-rich tandem repeat (TR) motif, PAPGSTAPPAHGVTSAPDTR, that is highly O-glycosylated in normal tissues. Moreover, the overexpression and aberrant glycosylation of MUC1 by tumour cells is correlated in cancer patients with the mounting of a

humoural immune response to a wide variety of cryptic MUC1 peptide and carbohydrate antigens resident in the TR. This finding has prompted interest in MUC1 analogues as potential antigens in the preparation of cancer vaccines. We report the biosynthesis and characterization of recombinant glycosylated MUC1 tandem repeat peptides harbouring the tumour-related Tn antigen. More precisely, MUC1 analogues, ranging in length from 5 to 20 TRs, were expressed in bacteria as isotopically labeled (15N-, 13C-) and subsequently glycosylated at 3 defined Ser/Thr positions within each tandem repeat using a recombinant human polypeptide N acetylgalactosaminyl transferase-T1 (rppGalNAc-T1) produced in Pichia pastoris. The specificity and activity of this enzyme was carefully dissected in real time, by performing 2D NMR experiments on the 15N, 13C-labeled mucin MUC1 peptides during the O-glycosylation step itself. Finally, we report the rapid isolation of a highly purified, His-tagged version of the rppGalNAc-T1 active domain (Supported by CBCRA/NCIC and PENCE).

(450) ***In vitro N*-glycosylation of Glycoproteins Using Refolded Glycosyltransferases**

Sami Saribas, Dan Bezila, Winnie Ngo, Pat Goode-Mason, Zakkee Brooks, Susann Taudte, Karl Johnson, Shawn DeFrees, Scott Willett and David Hakes

Neose Technologies Inc. 102 Witmer Rd. Horsham PA 19044.

Mammalian glycosyltransferases, involved in *N*-linked glycoprotein synthesis, are type II Golgi membrane glycoproteins. Although these enzymes have been successfully expressed in a variety of heterologous eukaryotic expression systems, production of high levels of active glycosyltransferases in bacteria has proved problematic. The Qasba laboratory has demonstrated expression of N-terminal deletions of bovine β-1,4-Galactosyltransferase (GalT1) as inclusion bodies, followed by efficient redox mediated refolding of active GalT1 [Boeggeman E, et al. Protein Expr. Purif. 30, 219-229]. We have obtained high levels of active maltose binding protein (MBP) fusions of N-terminal truncated bovine GalT1, rat α-1,3-sialyltransferase (ST3Gal3) and human *N*-acetylglucosaminyltransferase (GnT1), in *E. coli*, using redox mediated refolding of isolated inclusion bodies. While active, refolded enzyme was recovered using the native GalT1 and ST3Gal3 sequences, a mutation of an unpaired cysteine residue in human GnT1 was required for recovery of active enzyme. These three bacterially expressed, refolded enzymes were used in efficient glycan remodeling of a model glycoprotein substrate.

(451) **Synthesis of Sulfated Oligosaccharides by Sulfotransferase-transfected ECV304 Cells using Saccharide Primer and Structure Analysis by MALDI-TOF Mass Spectrometry**

Pei-Xing Wu[1], Naoko Kimura[2], Reiji Kannagi[2] and Toshinori Sato[1]

[1] Department of Biosciences and informatics, Keio University, Yokohama, [2] Program of Experimental Pathology, Aichi Cancer Center, Nagoya.

In the functional and structural analysis of carbohydrates for glycomics, one of the critical strategies is the facile synthesis of the desired oligosaccharide analogues. The development of efficient synthesis of oligosaccharides is an ongoing challenge and much progress has been made in recent years. We have so far embarked on biocombinatorial synthesis of oligosaccharides to construct an oligosaccharide library using cells in culture and saccharide primers. Using this method we have obtained more than 70 kinds of oligosaccharides including sialic acid-containing oligosaccharides and fucosylated oligosaccharides from various cell lines. Sulfated oligosaccharides are an important class of compounds in the field of glycobiology. However, sulfated oligosaccharides have not been detected from the cell lines employed so far. Expression of sulfated oligosaccharides in small quantity in biological systems has a problem for their biocombinatorial synthesis. On the other hand, mass spectrometry is a useful tool for the structural analysis of carbohydrates and offers precise results. Mass spectrometric analysis of sulfated glycoconjugtes is challenging due to their acidity and lability. Little information is available about structural analysis of sulfated carbohydrates by mass spectrometric analysis, especially by MALDI-PSD-TOF mass spectrometry. In this study, we employed GlcNAc:6-sulfotransferase-transfected ECV 304 cells and GlcNAc-C12 primer to produce sulfated oligosaccharides. Total lipids in culture medium were extracted by Sep-pak cartridge after incubation of cells with GlcNAc-C12 primer for 48 hours. The collected lipids were isolated by high performance thin layer chromatograph (HPTLC). The areas corresponding to orcinol-stained bands of the HPTLC plate were transferred to a PVDF membrane. The glycosylated products on the PVDF membrane were subsequently subjected to mass spectrometric analysis. Analysis by HPTLC of the lipids extracts from the culture medium indicated 9 bands corresponding to putative glycosylated products. MALDI-TOF mass spectrometric analysis showed that 4 kinds of glycosylated products possessed the molecular mass of sulfated oligosaccharide. These 4 kinds of sulfated oligosaccharides (S1~S4) were further analyzed by post-source decay spectrum. MALDI-TOF mass spectrum of S1 exhibited a peak at m/z 514.4 ([M+2Na-H]+) corresponding to a monosulfated primer. Three main fragment ions were observed in PSD spectrum indicating that this product was GlcNAcSO3-C12. MALDI-TOF mass spectrum of S2 revealed a peak at m/z 677.0 ([M+2Na-H]+) corresponding to a structure of Hexose-GlcNAcSO3-C12. The fragment ions in PSD spectrum are consistent with Hexose-GlcNAcSO3-C12. With the same methods, Structural analysis of S3 and S4 showed that S3 and S4 possessed the structure of Hexose-(Fucose)GlcNAcSO3-C12 ([M+2Na-H]+ = 823,04) and NeuAc-Hexose-GlcNAcSO3-C12 ([M+Na-2H]- = 943.9), respectively. In our work on biocombinatorial synthesis using mammalian cells and saccharide primers for constructing the oligosaccharide library, one of some efforts to be done is to generate oligosaccharides that are usually present in low quantity in cells. The present results also suggested that gene manipulation technology is a useful approach for our purpose to produce desirable oligosaccharides.

(452) **Neoglycolipid Macroarray to Characterize Specificity of Phage-displaying Antibodies against Carbohydrate Antigens**

Tomoki Chiba[1,2], Keiko Sakai[2,3], Munehiro Nakata[1,2,3], Atsushi Takayanagi[2,4] and Yoko Fujita-Yamaguchi[1,2,3]

[1] Dept. Appl. Biochem., Tokai Univ., [2] CREST JST, [3] Inst. Glycotech., Tokai Univ., [4] Mol. Biol., Keio Univ. Sch. Med., Japan.

INTRODUCTION: Involvement of specific carbohydrate structures in glycoconjugates with cell-cell recognitions and disease developments has been steadily understood. Antibodies against carbohydrate antigens are useful for detecting glycoconjugates bearing a specific carbohydrate structure. However, currently-available monoclonal antibodies (mAbs) against carbohydrate antigens are not good enough to detect a variety of carbohydrate antigens. Generation of large repertoires of phage-displaying antibodies and the following panning procedure permit the selection of antibodies having various specificity for carbohydrate structures. To screen a phage-displaying antibody for a distinct carbohydrate antigen, it is important to clarify the specificity of the antibody against carbohydrate structures. We have previously shown that neoglycolipids consisting of carbohydrates and dipalmitoylphosphatidylethanolamine (DPPE) are useful probes for characterizing lectin-carbohydrate interactions. In the present study, we report a method to characterize specific interactions between phage-displaying antibodies and carbohydrate antigens by means of neoglycolipids arrayed on solid phase. METHODS: Neoglycolipids were constructed with the conjugation of oligosaccharides and DPPE by reductive amination. Purified neoglycolipids were dissolved in appropriate solvents such as water and chloroform/methanol/water solvent systems. Phage-displaying antibodies against neoglycolipid bearing a trimannose core saccharide (Man3-DPPE) were prepared as follows. The library of filamentous bacteriophage M13 representing single chain antibody was subjected to four rounds of panning against Man3-DPPE immobilized in microtiter wells, from which positive clones were screened by ELISA using Man3-DPPE as an antigen. Relative affinity and specificity of phage-displaying antibodies were assessed with various neoglycolipids arrayed and immobilized on solid phases such as nitrocellulose, polyvinylidene difluoride (PVDF) and nylon membranes, or slica gel TLC plates. Solutions of neoglycolipids were dot-blotted onto those solid phases. The solid phases were blocked, overlaid with phage-displaying antibody solutions, and then incubated. After washing, bound phage-displaying antibodies were detected by the combination of anti-M13 mAb horseradish peroxidase conjugate and a chemiluminescent reagent. Similar binding assay was also performed with concanavalin A (Con A) horseradish peroxidase conjugates. RESULTS: PVDF membrane was not appropriate for dot-blotting of neoglycolipids since the spot size was uneven due to the solvent system in which neoglycolipid was dissolved. Nitrocellulose and nylon membranes and slica gel TLC plates were used for the construction of neoglycolipid macroarray, then subjected to the binding analysis of Con A or phage-displaying antibodies isolated by panning procedure and ELISA with Man3-DPPE. Binding of Con A to neoglycolipids bearing mannose residue(s) was observed on all solid phases tested. Binding of phage-displaying antibodies was, however, detected only on silica gel TLC plates. Each phage-displaying antibody tested was bound to Man3-DPPE, but some of them showed affinity not only to Man3-DPPE but also to neoglycolipids bearing mannnopentaose. DISCUSSION: This study suggests that neoglycolipid

macroarray presenting a variety of carbohydrate structures on silica gel TLC plates has the potential for analyzing specific interactions between phage-displaying antibodies and carbohydrates. Neoglycolipid macroarray would be one of the most useful tools for characterizing specificity of phage-displaying antibodies against carbohydrate antigens which should be applied for their basic research and clinical applications.

(453) **Screening of Single Chain Antibodies (scFvs) against a Variety of Carbohydrate Moieties**

Miyo Kimura[1,2], Keiko Sakai[2,3], Yoshitaka Shimizu[2,3], Tomoki Chiba[1,2], Munehiro Nakata[1,2,3], Atsushi Takayanagi[2,4], Nobuyoshi Shimizu[4] and Yoko Fujita-Yamaguchi[1,2,3]

[1] Dept. Appl. Biochem., Tokai Univ., [2] CREST JST, [3] Inst. Glycotech., Tokai Univ., [4] Mol. Biol., Keio Univ. Sch. Med., Japan.

INTRODUCTION: Antibodies generated against a variety of epitopes in peptides/proteins have provided invaluable tools for understanding structural-functional basis as well as developing clinical diagonosis kits in the last two decades. In contrast, structural-functional studies on carbohydrate moieties in glycoproteins and glycolipids remain extremely difficult due to their complex nature and the lack of highly sophisticated methodologies as compared to those for proteins. Antibodies against a variety of carbohydrate moieties would be extremely useful for precisely detecting glycoconjugates. Conventional methods of immunizing animals with carbohydrate antigens, however, have largely been unsuccessful in generating highly specific antibodies with high affinity against carbohydrate moieties. We have thus set our aim at producing a variety of single chain antibodies (scFvs) against structurally defined carbohydrate moieties, which should complement or even be superior to currently available lectins. To achieve this aim, we first constructed a phage-displaying human scFv library using "CDR shuffling" and "VL/VH shuffling" methods with unique vector constructs. Secondly, we prepared neoglycolipides by the conjugation of oligosaccharides and dipalmitoylphosphatidylethanolamine (DPPE) by reductive amination. Neoglycolipids were used to screen the phage display library representing over 10^{11} independent human scFvs. In this study, we present a first set of scFvs which we have isolated. METHODS: The phage-displaying human scFv library was subjected to four rounds of panning against trimannose-DPPE(M3-DPPE) or Le antigen-related carbohydrates conjugated to DPPE. Phage antibodies specific for these antigens were screened and identified by ELISA. Positive phage clones were analyzed by PCR, restriction enzyme digestion, DNA sequencing, and neoglycolipid macroarray. Further, scFv proteins were produced and assayed for their molecular weights by SDS-PAGE/immunoblotting and their specificities using neoglycolipid macroarray methods. RESULTS: Thus far, over 20 phage clones have been identified by ELISA using M3-DPPE as an antigen. Seven phage clones have been characterized, which indicated that six of them are independent clones. Further characterization of these clones is now in progress. Similarly, 16 phage clones against Le antigen-related carbohydrates have been obtained. Characterization of these phage antibodies are also in progress. DISCUSSION: This study suggests that an “in vitro” antibody production as we planned to achieve has a great potential in ultimately obtaining a variety of single chain antibodies against a variety of carbohydrate antigens, which will be, in turn, constructed to be antibodies useful for basic research as well as clinical application.

(454) **A Rapid and Sensitive Analysis of *N*-glycans as 9-Fluorenylmethyl chloroformateÅiFmocÅjderivatives by High-performance Liquid Chromatography with Fluorometric Detection: A Method Allowing to Recover Free Oligosaccharides after Analysis**

Satoru Kamoda[1,2], Miyako Nakano[1], Rika Ishikawa[2], Shigeo Suzuki[1] and Kazuaki Kakehi[1]

[1] Faculty of Pharmaceutical Sciences, Kinki University, Kowakae 3-4-1, Higashi-Osaka 577-8502, Japan, [2] KIRIN BREWRY Co., Ltd., Hagiwara-machi 100-1, Takasaki 370-0013, Japan.

There are a large number of labeling methods for asparagine-type oligosaccharides with fluorogenic and chromophoric reagents. We have to choose the most appropriate labeling method based on the purposes such as mass spectrometry, high-performance liquid chromatography and capillary electrophoresis. Asparagine-type glycans are released from core proteins as *N*-glycosylamine at the initial step of the releasing reaction when glycoamidase F is employed as the enzyme. The *N*-glycosylamine-type oligosaccharides thus released by the enzyme are subjected to hydrolysis or mutarotaition to form free-form oligosaccharides. In the detailed studies on the enzyme reaction, we found a condition in which the released *N*-glycosylamine-type oligosaccharides were exclusively present at least during the course of enzyme reaction. Thus, we developed a method to *in situ* derivatize the glycosylamine-type oligosaccharides with 9-fluorenylmethyl chloroformate (Fmoc-Cl). The Fmoc-labeled sialo- and asialo (or hi-mannose and hybrid)-oligosaccharides were successfully analyzed on an amine-bonded polymer column and amide-silica column, respectively. The present method showed approximately 5 times higher sensitivities than that using 2-aminobenzoic acid (2-AA). The separation profile was similar to that observed using 2-AA method as examined by the analyses of carbohydrate chains derived from several glycoproteins including complex-type, high-mannose type and hybrid type of *N*-linked oligosaccharides. The labeled oligosaccharides were stable at least for several months when stored at -20°C. Furthermore, it should be emphasized that the derivatized oligosaccharides could be easily recovered as free reducing oligosaccharides simply by incubation with morpholin in DMF solution. We obtained a pure triantennary oligosaccharide with 3 sialic acid residues as a free reducing oligosaccharide from fetuin in good yield after isolation of the corresponding Fmoc oligosaccharide followed by removing reaction of the Fmoc group. The proposed method will be quite useful for preparation of free oligosaccharides as standard samples at pmol-nmol scale from commercially available glycoproteins.

(455) **Proteomic Approach to Cancer-Associated Glycoproteins**

Masaya Ono, Tesshi Yamada and Setsuo Hirohashi

National Cancer Center Research Institute. 5-1-1 Tsukiji Chuo-ku Tokyo Japan.

Introduction: Cancer often carries a variety of alterations of carbohydrates in glycolipids and in N-linked or O-linked glycoproteins. Those alterations of carbohydrates not only serve as tumor markers, but also modify the function of glycoproteins (Ono, M.et al., Cancer Research 1999). Sialyl Lewis A is one of the representative carbohydrate tumor markers. High amounts of sialyl Lewis A defined by NS19-9 monoclonal antibody are present in human adenocarcinomas of the colon, pancreas and stomach. There are several lines of evidence showing that sialyl Lewis A regulates the adhesion of human cancer cells to E-selectin expressed in endothelium, and promotes metastasis. MUC1 mucin is known to carry sialyl Lewis A. However, the other proteins carrying sialyl Lews A remain largely unknown. In this study we took proteomic approach for the global identification of glycoproteins carrying the CA19-9 epitope. The functional modification of these proteins by cancer-associated glycosylation may shed lights on the novel aspect of cancer biology.
Material and method: Methyl-Éø-mannoside and methyl-Éø-glucoside-rich glycoproteins of BxPC3, a pancreatic cancer cell line expressing CA19-9, were enriched by the affinity column coupled with Concanavalin a (Con A). The Con A-binding fraction was further purified by immunoaffinity columns coupled with anti-CA19-9 antibody. Glycoproteins carrying the CA19-9 epitope were fractionated by SDS-PAGE. After carbaoxymethylation, the proteins were digested in-gel by modified trypsin, and extracted into 0.1% formic acid solution. The digested peptides were separated by nano-HPLC and detected by ESI-QTOF system. The data of MS/MS finger printing were analyzed by database search using the Mascot software with modification.
Result: 200Kd, 120-180Kd and 80-100Kd proteins were identified to carry the CA19-9 epitope by a simple two-step purification and blotting with antibody against sialyl Lewis A. The 120-180Kd protein was subject to identification by the nano-LC ESI-QTOF system. The mass fingerprints of the 120-180Kd protein matched to carcinoembryonic antigen (CEA). We confirmed CEA produced by BxPC3 cells contains the CA19-9 epitope by immunoprecipitation with anti-CEA antibody and immunoblotting with anti-CA19-9 antibody and vise versa.
Conclusion: For the purpose of identifying the glycoprotein bearing tumor-associated carbohydrate sialyl Lewis A, we conducted a proteomics approach and found that the CEA protein is one of those. Although CEA is also a widely used tumor marker, its functional significance remains unknown. Our approach represents an effective way to identify glycoproteins carrying functional carbohydrates. We are now investigating the 200Kd and 80-100Kd glycoproteins, and want to clarify the relationship between cancer biology and alterations in glycosylation.

(456) **Carbohydrate-mediated Gene Delivery System using a pDNA/Chitosan/Lactose-PEG-C Ternary Complexes**

Yohei Koya[1], Yoshiyuki Koyama[2] and Toshinori Sato[1]

[1] Department of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan, [2] Otsuma Women’s University, 12 Sanbancho, Chiyoda-ku 102-8357, Japan.

Various kinds of cationic polymers such as polyethyleneimine (PEI), poly-L-lysine (PLL) and chitosan have been widely explored as a safe and non-infectionous alternative to viral gene vectors. These polycations form electrostatic polyion complexes with plasmid DNA, and extremely enhance

the gene expression *in vivo* and *in vitro*. However, electrostatic interaction of cationic pDNA complex with cells is not desirable in the viewpoint of cellular specificity and targeting to a specific organ. One of the methodologies to make pDNA complex cell specific is to utilize carbohydrate-recognition. For example, hepatocyte and various cancer cell lines have affinity for galactose or lactose. In this study, we investigated receptor-mediated transfection of the ternary complex in which a pDNA/chitosan complex is coated with novel polyanions bearing cellular targeting moieties. As the novel polyanion, we synthesized lactose-modified polyethylene glycol derivative (Lac-PEG-C). This PEG derivative has both carboxylic acid and sugar side chains, and the anionic Lac-PEG-C can coat the surface of cationic pDNA/chitosan complexes. Zeta potential measurement showed that the particles of pDNA/chitosan/Lac-PEG-C (1:3:10 by charge ratio) became negative charge by coating pDNA/chitosan complexes (1:3 by charge ratio) with Lac-PEG-C. Dynamic light scattering (DLS) and atomic force microscopy (AFM) showed that the particle size of pDNA/chitosan/Lac-PEG-C complexes were about 200 nm as well as the pDNA/chitosan complexes. Although the pDNA/chitosan complexes aggregated time-dependently, the coating of the pDNA/chitosan complex with sugar-PEG-C depressed self-aggregation. The recognition function of galactose residues on the pDNA/chitosan/Lac-PEG-C complexes was investigated by RCA_{120}-induced agglutination. The agglutination of the ternary complex was significantly induced by the RCA_{120}, and was reversible by the addition of 100 mM lactose. Gene transfer efficiencies were evaluated by luciferase plasmid as reporter gene with HuH-7 cells that expresses asialoglycoprotein receptor. Compared with the transfection efficiency of pDNA/chitosan complexes (1:3 by charge ratio), that of pDNA/chitosan/PEG-C complex without lactose decreased due to the negative charge of the complex. On the other hand, coating of pDNA/chitosan complex with Lac-PEG-C resulted in three times increase of luciferase gene expression. The transfection efficiencies decreased to 1/10 by the addition of asialofetuin that is natural ligand of asialoglycoprotein receptor. These results indicated that pDNA/chitosan/Lac-PEG-C complexes were taken up by HuH-7 cells through receptor-mediated interaction. In this study, we developed the pDNA/chitosan/Lac-PEG-C ternary complexes and clarified their ability as a receptor-mediated gene delivery system. It is expected that the ternary complex would be an effective tool for targeting therapy *in vivo*.

(457) **Analysis of the Intracellular Transport of Plasmid Mediated by Chitosan**

Zhihong Yang and Toshinori Sato

Department of Bioscience and Informatics, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan.

Gene therapy involves the introduction of exogenous genes into target cells where production of the encoded protein will occur. For the advantage of no immunogenicity and low toxicity, there is an increased interest in the development of a non-viral gene delivery system that can circumvent the limitations seen on the viral approach. Polycation/DNA complexes are generally more stable than other non-viral gene delivery system, especially liposomal or cationic lipid systems. Chitosan is a natural cationic polymer consisting of polymeric 1-4 linked 2-amino-2-dexolyl- β-D-glucose and is derived from crustacean shells. Chitosan has been explored as a polycationic gene carrier in drug delivery system with promising results in the last few years, due to it`s good biocompatibility, low immunogenicity and low cytotoxicity. On the other hand, knowledge concerning the mechanism of the intracellular transport of plasmid /chitosan complexes is still relatively rudimentary. In this study, microinjection method was employed to elucidate it. After β-galactosidase plasmid DNA/chitosan complexes were injected into nucleus and cytoplasm separately with an Eppendorf micromanupilator FemtoJet system, the expression efficiency of exogenous β-galactosidase gene was estimated by counting the number of blue dyed cells. The effect of plasmid concentration, post-injection time and stoichiometry of plasmid/chitosan on transgene expression efficiency were investigated. Atomic force microscope (AFM), dynamic light scatter, and Zeta-potential measurement were employed to investigate the morphology, size and surface charge of pDNA/chitosan complexes of various N/P ratios used in this study. The results obtained from microinjection of free plasmid or plasmid/chitosn complexes into nucleus indicated that the complexation of plasmid with chitosan has no influence on the expression of pDNA, suggesting that it is not necessary for pDNA to release from pDNA/chitosan complexes before entering nucleus. The transcription and expression of pDNA/chitosan complexes in nucleus occurred very fast as well as pDNA. It is also suggested the nuclear import of pDNA/chitosan complexes was promoted by cell division. Microinjection into cytoplasm showed that chitosan protects pDNA in cytoplasm and promotes it`s transport into nucleus from cytoplasm, a step generally considered speed limiting. It has been reported that the optimum N/P ratio of pDNA/chitosan complexes was 5 for the expression of pDNA. N/P ratio is charge ratio of DNA (P) and chitosan (N). When the pDNA/chitosan complexes were microinjected in cytoplasm and nucleus, the optimum N/P ratio was also 5. These results suggest that chitosan play and important role for the compaction of pDNA and transport of pDNA into nucleus. In conclusion, it was indicated that chitosan is a useful biocompatible biopolymer to form compact nanoparticles with pDNA and promote the nuclear entry of pDNA

(458) **Determination of Sulfatides and Complicated Sulfated Glycosphingolipids by MALDI-TOF MS**

Mamoru Kyogashima[1], Atsushi Hara[2], Toshifumi Aoyama[2] and Reiji Kannagi[1]

[1] Aichi Cancer Center Research Institute, Nagoya Japan, [2] Shinshu University Graduate School of Medicine, Matsumoto Japan.

Sulfatide (the sulfuric ester of galactosylceramide at the C3 of the galactose, SM4s) variously influences the diseases related to vascular involvements such as thrombosis, atherosclerosis and cancer metastasis. The involvement of sulfatide in blood coagulation and haemostasis has long been suggested. In fact, recently we demonstrated that in the presence of severe vascular injury, sulfatide strongly accelerates thrombogenesis but that in the absence of the injury, sulfatide behaves anticoagulantly (1). Leukocytes, platelets and endothelial cells express sulfatide on their surfaces. It is major glycosphingolipid in all kinds of serum lipoproteins and is accumulated in the atheromatous lesions of aorta. Tumor cells, such as lung cancer cells or leukemia cells, highly express sulfatide and further some of them shed sulfatide, which may bind many sulfatide binding proteins, such as laminin thrombospondin and von Willebrand factor in extracellular matrix surrounding vascular vessels. Therefore, we tried to determine the sulfatide with high accuracy and sensitivity in order to clarify the role of sulfatide in the diseases, which sulfatide may concern. Many monoclonal antibodies against sulfatide have been generated. However their specificity may not always be satisfactory and accordingly the reports sometimes contradicted each other. Furthermore the antibodies can not clarify the ceramide structure, which may significantly influence the circumstances and the functions of the sulfatide. To overcome these problems, we applied matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). We tried micro-scale analyses of sulfatides from pig spinal cord and rat kidney. The former mainly contained ceramides as d18:1 or d18:0 with C24:1, d18:1 or d18:0 with C24:0, and d18:1 or d18:0 with C24:0h, and the latter mainly contained those as t18:0 with C24:1, t18:0 with C22:1 and t18:0 with C24:0. Thus obviously sulfatides are composed of different ceramide structures according to their origin. Furthermore, from rat kidney, we could determine a complicated sulfated glycosphingolipid, SM2 (mono-sulfated gangliotriaosylceramide), having ceramides as t18:0 with C24:0, t18:0 with C22:0 and t18:0 with C23:0h. (1) M. Kyogashima, The role of sulfatide in thrombogenesis and haemostasis. Arch. Biochem. Biophys. 426, 157-162 (2004)

(459) **Structural Analysis of Stereo-isomers of Fuc α/β (1→6)Gal α/β (1→6)Glc α/β Octyl using ESI-ITMS^n**

Shusaku Daikoku, Takuro Ako and Osamu Kanie

11 Minamiooya, Machida-shi, Tokyo 194-8511, Japan.

Introduction Oligosaccharides present on the cell surfaces of multicellular organisms play important roles in many biological processes. Oligosaccharide covalently linked to proteins is forming large array of molecular family as glycoprotein. These structures are synthesized by sequential reactions of multiple enzymes such as glycosidases and glycosyltransferases. Thus the structure of oligosaccharide cannot be identified by reading the nucleotide sequence. Hence, structural analysis of oligosaccharide has to deal with a minute amount of isolated materials from biological samples. Analysis based on mass spectrometry offers several advantages over other techniques: speed of analysis, high sensitivity at low picomol level, etc. The sequencing of peptide can be achieved by existing analytical method based on mass spectrometry. Oligosaccharides consist of regio-isomers, stereo-isomers, so-called anomers, and branchs, where the peptides consist of linear amide bonds. The structure of oligosaccharides therefore cannot be determined in this manner. We investigated MSn analysis using ESI-ITMS of trisaccharide consists of a series of anomers, where intensity of individual fragment ions were obtained by varying amplitude of CID voltage. *Experimental* At first, an anomeric mixture of

Structural analysis of stereo-isomers of Fuc α/β (1→6)Gal α/β (1→6)Glc α/β Octyl using ESI-ITMS^n was separated using LC-ESI-MS (Waters). Individual samples were analyzed by using a quadrupole ion trap mass spectrometer equipped with electrospray interface (Bruker Daltonics esquire 3000 plus). The molecular ions of trisaccharides, Structural analysis of stereo-isomers of Fuc α/β (1→6)Gal α/β (1→6)Glc α/β Octyl using ESI-ITMS^n, were all observed at m/z 623 as soduium adducts. The MSMS analysis of precursor ions of m/z 623 were carried out where RF amplitude was varied from 0.5V with 0.02V increments until precursor ion disappeared. Fragment ions with m/z 477 were observed for all samples. The fragment ions were the products of fucosyl cleavage at the non-reducing end. The obtained graph from MSMS analysis suggested Éø-fucosyl linkages were more labile when compared with corresponding É¿-linkages. Furthermore, MSMSMS analysis of ion with m/z 477 as a precursor revealed that the ions with identical m/z possibly having the same structures resulted in a same graph. It was clearly shown that our findings are very useful in a sequencing of oligosaccharide based on mass spectrometry. *Acknowledgements* This research was supported by Key Technology Research Promotion Program, The New Energy and Industrial Development Organization (NEDO), Ministry of Economy, Trade and Industry (METI) of Japan.

(460) A Fragmentation Database for Electrospray Mass Spectroscopic Profiling of Keratan Sulfate Relating Oligosaccharides

Toshikazu Minamisawa[1,2], Kiyoshi Suzuki[2] and Jun Hirabayashi[1]
[1] Glycostructure Analysis Team, Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), Central-6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8586, Japan, [2] Central Research Laboratories, Seikagaku Corporation, 3-1253 Tateno, Higashi-yamato, Tokyo 207-0021, Japan.

Keratan sulfate, a group of glycosaminoglycans having a linear backbone consisting of alternating galactose and *N*-acetylglucosamine residues, is predominantly found in cornea and cartilage. All of hydroxyl groups at the C-6 position of *N*-acetylglucosamine are sulfated, whereas the extent of sulfation of hydroxyl groups at the C-6 position of galactose significantly varies in origins. Further modifications with sialic acid (i.e., capping at non-reducing end) and fucose (i.e., branching) also reflect the difference in their sources. To overcome such complex features of keratan sulfate for structural analysis, development of a facile, sensitive and reliable methodology is necessary. Electrospray ionization ion trap mass spectrometry (ESI-IT-MS) should meet the requisites.

For this purpose, more than 10 oligosaccharides derived from keratan sulfate, having *N*-acetylglucosamine at reducing end and various contents of sulfate groups, of which structures were defined, were analyzed. They were dissolved at 10 nmol/mL in 50% methanol containing 1 mM acetic acid, and were directly infused into an ESI-IT-MS apparatus (Esquire 3000 plus, Bruker Daltonics) via a syringe at a flow rate of 360 µl/hr. All spectra were acquired in a negative ion mode and serial collision induced dissociation MS^n experiments were performed.

Under the experimental conditions we employed, fully charged ions, reflecting the number of sulfates and sialic acids in the oligosaccharide structures, were observed as dominant peaks in all MS^1 spectra. In subsequent MS^n experiments, almost all fragment ions derived from each of the precursor ions could be successfully assigned. Since individual oligosaccharides gave characteristic fragmentation patterns depending on their sulfation features, they were clearly differentiated from one another. Apparently, there was some relationship between their structures (with different sulfations) and their fragmentation patterns. As a result, their structures were successfully profiled with no derivatization procedure. Therefore, this kind of MS^n approach is promising for elucidation of important empirical rules as well as construction of a structural database as regards sulfated oligosaccharides derived from glycosaminoglycans under the concept of glycan profiling. Based on the developed protocol, we also analyzed partial digests of both corneal and cartilaginous keratan sulfates.

This work is partly supported by the New Energy and Industrial Technology Development Organization (NEDO) in Japan.

(461) Quantitative Analyses of Maltooligosaccharides by Collision Induced Dissociation using Ion-Trap Mass Spectrometry

Ayako Kurimoto, Shusaku Daikoku and Osamu Kanie
Mitsubishi Kagaku Institute of Life Sciences (MITILS) 11 Minamiooya, Machida-shi, Tokyo 194-8511, Japan.

Introduction

Oligosaccharides exist as conjugates of lipids and proteins in living organisms and involved in most of biological functions including cell-cell and cell-matrix recognition, hormonal actions, inter- and intracellular trafficking and protection. Unlike DNA and proteins where sequence provides nearly all the primary structure, oligosaccharides can form a large number of structural isomers, linear sequence, branching, stereoisomers and positional isomers. Addition to the diversity of oligosaccharides, they cannot be amplified by the state-of-the-art biotechnological methods, e.g. PCR, which makes the structural elucidation of oligosaccharides significantly more difficult than that of other biopolymers (peptides, proteins and oligonucleotides). The lack of a rapid method for the complete structural elucidation of oligosaccharides remains a major problem in understanding strucrture-function relationships. The importance of the new sequencing methods for carbohydrates is increasingly being recognized. In this investigation, we utilized electrospray-ionization ion-trap mass spectrometer (ESI-ITMS), which has been widely recognized as an effective means for studying the details of oligosaccharides. This technology presents the ability to perform multiple stages of fragmentation (MS^n). Recent advances of quadrupole ion-trap mass spectrometry (QIT-MS) equipment are allowing us to obtain spectrum of collision-induced dissociation (CID) at MS^n. The CID was shown to be powerful means for the structural analysis of biomolecules and oligosaccharides. Based on the recognition of the importance of CID technique, we have examined quantitativity of CID-spectra at different stage of MS^n. One of the problems in determining isomeric structures associates with lack of quantitative information in mass spectra obtained by CID. As an example, maltoologosaccharides were used to obtain spectra.

Experimental

Structural analysis of maltooligosaccharides was carried out using electrospray ionization with an esquire 3000^{plus} ion trap mass spectrometer (Bruker Daltonics Corp.) at positive ion mode. 2-Aminopyridine (PA)-derivatized malto-pentaose, hexaose and heptaose were prepared by reductive amination. These samples were dissolved in methanol/water solution and infused by syringe pump and nebulized with dry nitrogen. The CID spectra were obtained using helium as a damping gas.

Results

In order to obtain vital information about both m/z and quantity of fragment ions, we examined CID spectra at different stages of MS^n of PA-maltooligosaccharides. The $[M+Na]^+$ ion of PA-maltoheptaose (m/z 1253) was subjected to CID with increasing RF voltage, and generated Y ions at m/z 1091 (Y_6) and m/z 929 (Y_5), which were then subsequently mass-selected and fragmented at MS^3. The CID data of MS^2 experiment of PA-maltohexaose (m/z 1091) and that of MS^3 experiment of m/z 1091 yielded from PA-maltoheptaose were in good agreement regarding fragmentation pattern i.e. m/z and yielding curves of daughter ions. The CID pattern of MS^2 of PA-maltopentaose (m/z 929) was very similar to that of m/z 929 from PA-maltoheptaose as well. The results indicated that when a trapped ion species is same, fragmentation and spectra are identical even at different stages of MS^n, indicating information obtained in CID experiments might be discussed quantitatively.

Acknowledgements

This research was supported by Key Technology Research Promotion Program, The New Energy and Industrial Development Organization (NEDO), Ministry of Economy, Trade and Industry (METI) of Japan.

(462) Galα1-4Gal (galabiose) on Glycoproteins are Expressed in the Majority of Avian Species

Noriko Suzuki[1], Michael Laskowski, Jr.[2] and Yuan C. Lee[1]
[1] Department of Biology, The Johns Hopkins University, Baltimore, MD, 21218, [2] Department of Chemistry, Purdue University, West Lafayette, IN, 47907.

Glycoproteins containing Galα1-4Gal (galabiose) were thought to be rarely found in vertebrates except in a few species of birds and amphibians. In mammals, Galα1-4Gal sequence is only found in glycolipids (e.g., Gb3, Galα1-4Galβ1-4Glc-Cer) expressed on cell surfaces, and is a minimum structure recognized by several bacterial adhesins (e.g., P-fimbriae of uropathogenic *Escherichia coli*) and enterotoxins (e.g, Shiga toxin).

Pigeon (*Columba livia*) egg white and serum glycoproteins are rich in Galα1-4Gal-terminated *N*-glycans. Galα1-4Gal is also reported to be present in turtle-dove, budgerigar, cockatiel, and swiftlet, while glycoproteins containing Galα1-4Gal had not been found in chicken, quail, turkey, pheasant, duck and gander. To systematically investigate the distribution of (Galα1-4Gal)-expression in avian species, we examined the presence of (Galα1-4Gal)-glycoproteins in egg whites from 20 orders, 88

families, 163 genera, and 181 species of birds, as probed by western blot with *Griffonia simplicifolia*-I lectin (terminal α-Gal/GalNAc-specific) and anti-P_1 mAb (Galα1-4Galβ1-4GlcNAcβ1--specific). One of the significant observations is the total absence of (Galα1-4Gal)-glycoproteins in Ratitae (e.g., ostrich, tinamou) and Galloanserae (e.g., chicken, duck, curassow), which are phylogenetically separated from other orders at earlier stage of modern bird diversification (100--65 million years ago). Presence or absence of (Galα1-4Gal)-glycoproteins in other avian orders varied by the species (104 species positive, 44 species negative), even though some of them belong to the same order or family. There seem to be more avian species expressing Galα1-4Gal than those without it. Our results revealed that the expression of (Galα1-4Gal)-glycoproteins was most likely differentiated at earlier stage of diversification in modern birds, but some species of birds belonging to non-Ratitae/Galloanserae might have lost ability to express (Galα1-4Gal)-glycoproteins in egg white as recent as during or after diversification of avian families or genera (65 million years ago--present). Although the expression ability of (Galα1-4Gal)-glycoproteins may not be indispensable for all avian species, it had been acquired and maintained in some birds presumably for protecting themselves against pathogenic microbes.

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(463) The Carbohydrate Sequence Markup Language (CabosML): a XML Description of Carbohydrate Structures

Norihiro Kikuchi[1], Akihiko Kameyama[2], Shuuichi Nakaya[2,3], Hiromi Ito[2], Takashi Sato[2], Toshihide Shikanai[1,2], Yoriko Takahashi[1] and Hisashi Narimatsu[2]

[1] Mitsui Knowledge Industry Co., Ltd., Nakano-ku, Tokyo, Japan, [2] Glycogene Function Team, Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki, Japan, [3] Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan.

Bioinformatic resources for genomics and proteomics are widely available for molecular biologists, but this situation has not been achieved yet for glycobiologists. The complexity of carbohydrate sequences makes it difficult to define a common language to represent carbohydrate sequences and to develop bioinformatics tools for glycomics. In this study, we developed a carbohydrate sequence markup language (CabosML), a XML (extensible markup language) description of carbohydrate structures, and implemented a structural database of carbohydrates with the XML format. We also developed graphical user interfaces for editing and searching the carbohydarate structures, by which the depicted structures can be automatically converted into the XML formats. The XML description for carbohydrates described here will greatly contribute to the progress of informatics tools for glycomics.

(464) Design and Functional Evaluation of Photoaffinity Labeling Reagents Having Fluorescent Glycopeptides

Suzuki Masaya, Ohguro Yuuya, Nishida Yoshihiro and Kobayashi Kazukiyo

Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan.

Key Word: Fluorescent probe / β-Peptide / Artificial glycopeptide / Photoaffinity labeling

Introduction

Affinity labeling is a powerful tool to reveal the interaction between a protein and a ligand. Though many affinity labeling reagents have been developed, many of them have a difficulty of detecting and separating of labeled proteins and peptides. Recently, we developed a new class of β-peptide skeleton (5-amino-1,3-benzodioxole-4-carboxylic acid) as a fluorescent linker.[1)] In the present study, as application of the fluorescent peptide, we designed fluorescent photo affinity labeling reagents, in which glycosides (α-mannoside, α-trimannnoside and β-lactoside) and photo reactive groups were introduced at C-terminus and N-terminus of the fluorescent β-peptide, respectively.

Result and Discussion

First, we evaluated fluorescent properties of the labeling reagents. The reagents possessing a 4-phenylazide group at N-terminus increased the fluorescence upon photo reaction, though the reagents *per se* showed little fluorescence. This fluorescent property is very useful to detect labeled proteins and peptides. While the reagents possessing a trifluoromethyldiazirine had little fluorescence and showed no fluorescent response. In this case, strong electron-withdrawing of the CF_3 group may bring about the fatal loss of fluorescent emission energy.

Second, a mixture of WGA and ConA lectins were photo labeled with the β-lactoside and α-trimannoside photo affinity labeling reagents possessing a 4-phenylazide group. Expectedly, the respective lectin was labeled only with the corresponding specific reagent, and the labeled lectins were detected and separated as fluorescent bands by SDS-PAGE. Moreover, the fluorescent glycosyl labeled lectins were purified simply by affinity column chromatography and obtained as fluorescent protein crystals.

In addition, the reagents are useful for the preparation of fluorescent glycomaterials. We prepared carbohydrate immobilized silica particles having fluorescence and evaluated their functions with lectin staining by fluorescent microscopy.

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(465) Mammalian PIG-X and Yeast Pbn1p are the Regulatory Components of ER-Resident GPI-mannosyltransferase I

Hisashi Ashida[1], Yeongjin Hong[2], Nakaba Sugimoto[3], Yoshiko Murakami[1], Yusuke Maeda[1] and Taroh Kinoshita[1]

[1] Research Institute for Microbial Diseases, Osaka University, Japan, [2] Chonnam National University Medical School, Korea, [3] Graduate School of Medicine, Osaka University, Japan.

Within the endoplasmic reticulum (ER), Man and Glc donated from dolichol-phosphate (Dol-P)-Man and Dol-P-Glc are transferred to GPI-anchor and N-glycan precursors, and Ser residues in many proteins. Glycosyltransferases that mediate these reactions are ER-resident multi-transmembrane proteins with common characteristics, forming a family of more than ten enzymes. Previously, we cloned the gene, termed *PIG-M*, for GPI-mannosyltransferase-I (GPI-MT-I), which transfers the first of the four Man residues in the GPI-anchor precursors (1). Here, we newly isolated GPI-anchored protein (GPI-AP) deficient mutant CHO2.46 cells using GPI-recognizing bacterial toxins: *Aeromonas hydrophila* proaerolysin and *Clostridium septicum* alpha-toxin. CHO2.46 cells were found to be defective in GPI-MT-I activity but were not restored by transfection of *PIG-M* cDNA. We cloned the gene responsible, *PIG-X*, from rat cDNA library by expression cloning. Rat *PIG-X* gene uses an unusual CTG initiation codon and the ORF encodes 252 amino acid polypeptide. Analysis of N-terminal sequence of the mature PIG-X revealed that N-terminal 22 amino acid residues were processed. One transmembrane domain was predicted near its C-terminus. Two N-glycosylation sites in the N-terminal hydrophilic region were glycosylated with Endo-H-sensitive N-glycans. These observations indicated that PIG-X is an ER-resident type-I transmembrane protein with a large lumenal domain. FLAG-tagged PIG-X and GST-tagged PIG-M expressed in wild-type CHO cells were co-precipitated, suggesting that PIG-X and PIG-M formed an enzyme complex. The expression level of PIG-M was less than 10% in the absence of PIG-X, indicating that PIG-X stabilizes PIG-M. We found that *S. cerevisiae* Pbn1p (YCL052C), which had been reported to be involved in autoprocessing of pro-proteinase B in the ER (2), has sequence similarity with PIG-X. The transfection of CHO2.46 with *PBN1* alone did not restore the surface expression of GPI-AP, whereas co-transfection of *PBN1* and *GPI14* (YJR013W), the yeast homologue of *PIG-M*, complemented the expression to a level comparable to that restored by rat *PIG-X*. Moreover, the combination of *PBN1* and *GPI14* restored the surface expression of GPI-AP on human PIG-M mutant Ramos 517-17 cells, although each of the two alone did not. These results clearly indicates that Pbn1p is the functional homologue of mammalian PIG-X and that the association of PIG-X and PIG-M is not interchangeable between mammals and yeasts. This is the first report of a regulatory subunit of Dol-P-Man/Glc utilizing glycosyltransferases.

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(466) **Molecular Cloning and Characterization of endo-α-*N*-acetylgalactosaminidase from *Bifidobacterium longum***

Fusako Oura[1], Noriko Nagamine[1,2], Kiyotaka Fujita[1], Takane Katayama[1,2], Hidehiko Kumagai[1] and Kenji Yamamoto[1,2]
[1] Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, [2] CREST.

Endo-α-*N*-acetylgalactosaminidase (endo-α-GalNAc-ase, EC 3.2.1.97) catalyzes the hydrolysis of *O*-glycosidic α-linkages between Galβ1,3GalNAc and Ser/Thr residues of mucin-type glycoproteins. The enzyme has been purified from the culture filtrates of various microorganisms such as *Clostridium perfringens, Streptococcus pneumoniae, Alcaligenes* sp., *Streptomyces* sp., and *Bacillus* sp., and their enzymatic properties have been determined in detail. However, no structural and genetic information is available because of the difficulty in preparing a large amount of the enzyme. Recently, we found that *Bifidobacterium longum* JCM1217 produced endo-α-GalNAc-ase and it seems likely that the bacterium is a good target for elucidating a role of this enzyme in bacterial colonization of intestine abundant in mucin. In order to isolate the gene encoding endo-α-GalNAc-ase, we scrutinized several genomic DNA databases including that of *B. longum* NCC2705, and found that one ORF of unknown function with a predicted signal peptide at the N-terminus was highly conserved in the genomes of bacteria reported to secrete endo-α-GalNAc-ase. Then, the gene was amplified by PCR using the genome of *B. longum* JCM1217. The amplified gene was inserted into pET23d vector in order to be expressed as a hexahistidine-tagged protein, introduced into *E. coli* BL21 (λDE3), and then, the recombinant protein expressed was purified by a nickel-affinity column chromatography. The purified protein was found to liberate disaccharide Galβ1,3GalNAc from the chromogenic substrate Galβ1,3GalNAcα1-*p*NP, which was revealed by ^{1}H NMR and FAB-MASS analyses, demonstrating that the cloned gene indeed encodes endo-α-GalNAc-ase. The protein consists of 1,966 amino acid residues with a signal peptide and a transmembrane anchor at N- and C-termini, respectively. The deduced amino acid sequence did not exhibit significant similarity to those of any glycoside hydrolase (GH) families, but showed high similarity to those of ORFs (unknown function) of *Clostridium perfringens, Streptococcus pneumoniae, Streptomyces coelicolor*, and *Enterococcus faecalis*. This fact strongly suggests these enzymes constitute a novel GH family. The recombinant enzyme of *B. longum* JCM1217 had a strict substrate specificity, exclusively acting on core 1 structure, but no action at all on core 2 and core 3 structures of mucin-type sugar chains. Optimal pH and temperature of the enzyme were determined to be 5.0 and 60Åé, respectively, and the enzyme was stable below 37Åé. The *Km* and *kcat* values were estimated to be 0.16 mM and 37 s^{-1} , respectively. ^{1}H NMR analysis revealed that the hydrolysis reaction proceeds through the retaining mechanism. Like other retaining glycosidases, the recombinant enzyme could transfer the sugar moiety of the substrate to various monosaccharides (transglycosylation), as revealed by HPLC and MALDI-TOF-MS analyses. To identify the catalytic pair (general acid-base and nucleophile), the primary structures of the homologous proteins were aligned and compared. Then, 22 highly conserved carboxylic residues (Asp and Glu) were selected as the candidates and replaced with Asn and Gln residues, respectively, using site-directed mutagenesis. When the generated mutants were examined for decrease in hydrolysis activity, drastic decrease was observed for two mutants, and their properties are now being investigated.

(467) **A Xylanase Carbohydrate Binding Module as a Scaffold for Molecular Diversity**

Lavinia Cicortas Gunnarsson[1], Eva Nordberg Karlsson[2], Ann-Sofie Albrekt[1], Mats Andersson[3], Olle Holst[2] and Mats Ohlin[1]
[1] Department of Immunotechnology, Lund University, PO Box 7031, S-220 07 Lund, [2] Department of Biotechnology, Lund University, PO Box 124, S-221 00 Lund, [3] Alligator Bioscience, Scheelev‰gen 19A, S-223 70 Lund, Sweden.

The growing field of biotechnology is in a constant need of binding proteins with novel specificities and affinities. For large-scale applications, additional properties such as structural stability and high productivity are also required. In order to find such molecules, libraries are created by diversifying naturally occurring binding proteins, such as antibodies, which in those cases serve as scaffolds. In this way specific binders have been successfully evolved towards different target molecules although it has often proven difficult to raise specific binders against one type of ligands, that of carbohydrates. In this study we investigated the use of a thermostable carbohydrate binding module, CBM4-2 from a xylanase found in *Rhodothermus marinus*, as a diversity-carrying scaffold. It is a well-characterised module with a molecular weight of 18 kDa, a known beta jelly-roll structure and defined substrate affinity and specificity. The fact that it has a relatively unusual capacity to bind both to xylan and to amorphous cellulose, although the affinity for the latter is much lower, raised the possibility that additional changes in the binding site could further diversify the binding specificity of CBM4-2. A combinatorial library was created by introducing restricted variation at 12 positions in the carbohydrate binding site of the CBM4-2. Despite the small size of the library, variants specific towards different carbohydrate polymers (birchwood xylan, Avicel and ivory nut mannan) as well as a glycoprotein (human IgG4) were successfully selected for, using the phage display method. Investigated clones showed a high productivity (on average 69 mg purified protein/l shake flask culture) when produced in *Escherichia coli* and they were all stable molecules displaying a high melting transition temperature (75.7±5.3∞C). All our results demonstrate that the CBM4-2 molecule is a suitable scaffold for creating specific binders that also possess properties useful in different biotechnological applications. Our next goal is to evolve binders specific towards biomedically important glycans and glycoproteins and work is already on progress to achieve that.

(468) **Single Cell Glycosyltransferase Assay using Capillary Electrophoresis with Laser-induced Fluorescence Detection**

Leon H. Lau, Glen K. Shoemaker, Justin Lorieau and Monica M. Palcic
Department of Chemistry, University of Alberta, Edmonton, Canada.

Single cell analysis is preferred over bulk cell analysis as it allows for differences between individual cells to be detected rather than averaged with other cells. Furthermore, bulk cell analysis cannot be used to study cooperative activities such as quorum sensing nor can it be used to analyze small samples available in developmental studies or tumor biopsies. Glycosyltransferases are integral in the glycosylation pathway which plays a critical role in intercellular recognition and adhesion as well as intracellular regulation of cell development, differentiation and apoptosis. In the present work, the blood group A synthesing enzyme N-acetylgalactosaminyltransferase (GTA) from HT29 colon cancer cells was used to develop an ultra-sensitive micro-volume assay with sampling at various time points with a nanopipettor.

A novel nanopipettor was developed from inexpensive components that can deliver a reproducible volume over a range of nanoliter-sized volumes. This device was used to repeatedly sample 25 nL from a micro-reaction chamber containing approximately 200 nL of a reaction mixture containing a single HT29 cell, UDP-GalNAc, and H Type II acceptor labeled with the fluorescent tag tetramethylrhodamine (TMR). The blood group A antigen product was separated from the H antigen using micellar electrokinetic capillary chromatography and detected by laser-induced fluorescence. This is a powerful analytical technique as it combines the extraordinary separation ability of capillary electrophoresis with the sensitive detection offered by laser-induced fluorescence of the TMR label. The results obtained indicate that HT29 cells are heterogeneous in terms of GTA expression. Of the single cells assayed, only a portion had GTA activity. This is consistent with flow cytometry data observed by the authors as well as by Ichikawa et al. Int. J. Cancer 76:284 (1998). The methods used to study this glycosyltransferase in HT29 cells can be applied to other cells to observe any enzymatic reaction as long as a fluorescently labeled substrate is available. They can also be adapted to study purified enzymes where the activity is too low to be studied by conventional methods such as ELISA and radiochemical assays.

(469) **Development of a Quantitative Real-time PCR Method for Analyzing Transcript Abundance of Glycan-Related Genes in Mouse Tissues and Embryonic Stem Cells**

Alison V. Nairn and Kelley W. Moremen
The Complex Carbohydrate Research Center at the University of Georgia, Athens, GA 30602.

Quantitative real-time (kinetic) RT-PCR (kRT-PCR) is an emerging technique for determining transcript abundance at the cellular level. Other

techniques, such as serial analysis of gene expression (SAGE) and microarrays, are also commonly used to evaluate transcript levels, but kRT-PCR is especially useful for detecting low abundance transcripts. The limitations of the kRT-PCR technique entail difficulties in scaling up for the analysis of several hundred genes from a single RNA sample. The necessity for gene-specific primer design and large scale PCR analysis with appropriate validation and standardization has presented a significant hurdle for general use of kRT-PCR for high-throughput transcript analysis. We are developing a technique that can be adapted to robotic mid- to high-throughput data collection for transcript analysis of glycan-related genes in mouse cells and tissue samples, including embryonic stem cells at various stages of differentiation. The approach employs the intercalating fluorescent dye SYBR Green to detect the cDNA amplification products using gene-specific primer pairs without the need for more expensive fluorescent probes or beacons. Effective primer design is a critical element for obtaining consistent data with kRT-PCR. In this study, we have used mouse genomic DNA as a template to validate primer pairs designed for several mouse sialyltransferase genes [Family GT29 (CAZY, http://afmb.cnrs-mre.fr/CAZY/)]. The use of genomic DNA as a control template source has allowed us to test the amplification efficiencies of primers without the necessity for isolating cloned versions for each of the selected genes to validate the primer design. Various parameters of primer design were examined, including primer length, Tm, amplimer length and amplimer secondary structure. Test amplifications using genomic DNA and gene-specific primer pairs were evaluated using amplification efficiency and melt curve analysis to confirm that a single high efficiency product was being amplified. Our data suggest that a narrow window for primer length (19-21bp), primer Tm (60±1ʃC) and amplimer length (65-75 bp) results in primers with good amplification efficiencies (100± 10%). In the event that amplification efficiencies were outside of the acceptable range (<80% or >110%) or if more than one product was detected from the melt curve analysis, another primer set for that gene was tested. Further studies are underway to test the parameters for RNA isolation, cDNA synthesis, and optimization of robotic assembly of reaction mixtures in order to define the most effective protocols for medium-throughput transcript analysis of glycan-related genes in mouse cells and tissues. Our poster will present data on transcript abundance for several representative glycosyltransferase families in several mouse tissues using our optimized kRT-PCR protocols.

(470) **Optimization of TLC/VC MALDI-FTMS Method for Ganglioside Analysis.**

Vera B Ivleva, Bogdan A Budnik, Peter B O'Connor and Catherine E Costello

Boston University School of Medicine, Boston, MA.

Introduction Our previously developed method of direct coupling of thin layer chromatography (TLC) with Vibrationally-Cooled (VC) MALDI-FTMS allows simple handling of TLC with FT high resolution which is not affected by the irregular surface of the TLC plate. Use of vibrational cooling is necessary for ganglioside stabilization and detection. We now show that optimization of this technique leads to high sensitivity for the ions desorbed directly from the TLC plate. Furthermore, accumulation of the ions prior to transferring them into the cell allows application of an internal calibration technique for accurate mass assignment. Methods The ganglioside standards were separated by thin layer chromatography and the positions of the spots were determined prior MS-analysis. The sinapinic acid matrix solution was sprayed onto the TLC plate and the gangliosides were MALDI-desorbed directly from the TLC plate surface. MALDI-FTMS experiments were carried out on our home-built FT-ICR mass spectrometer with 7T magnet and Nd:YAG (UV) laser, frequency tripled to 355 nm, and Er:YAG (IR) laser (2940 nm). The collision cooling gas raises the pressure in the ion source during the desorption-ionization event. Internal Calibration on Adjacent Samples (InCAS) was performed using peptide standards desorbed from the stainless steel plate as calibrants. Preliminary results The sensitivity obtained for the gangliosides desorbed from TLC was 1 pmole (total amount spotted on a TLC plate prior to separation). The InCAS demonstrated that accuracy is not affected upon desorption of the analyte from uneven TLC plate surface. The accuracy of 0.3-1.5 ppm was obtained for five ganglioside standards. The effect of several parameters on molecule stabilization were studied, such as the type of the cooling gas, gas pulsing coordinating with ionization-desorption event, cooling gas pressure, duration of gas pulse, different laser intensities, focusing and beam profile. Soft IR-desorption of the gangliosides with urea as the matrix, together with 'cooling' in the Vibrationally Cooled source eliminated sialic acid loss. However, the matrix effect on the extent of the ganglioside fragmentation was significant, as was demonstrated by the comparison of desorption using urea as a matrix versus desorption directly from the graphite-coated stainless steel plate. IR-MALDI of the gangliosides directly from the TLC plate was not as efficient as UV-MALDI and the sensitivity was relatively low, apparently because the matrices were poorly incorporated into the bulk of the TLC plate surface, due to their crystallization patterns. Modifications of the ion source that will decrease the pump-down time are now underway, and these should allow use of glycerol as a TLC-compatible IR-matrix that will be able to penetrate into the silica surface of the TLC plate. The developed method is being applied to the samples from biological systems such as whole brain ganglioside mix, Leishmania lipids, and ganglioside rich lipid rafts from neuronal cells. Since the large area XY stage allows facile coupling to many surface techniques - not only TLC, but also polyacrylamide gel electrophoresis (PAGE) and surface plasmon resonance (SPR)- future studies will extend the range of applications to these analytical methods as well.

(471) **Multiple Sampling in Single Cell Analysis: α-glucosidase II Assays and Beyond**

Glen K Shoemaker, Leon H Lau, Justin Lorieau and Monica M Palcic

Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2G2.

The single cell, which represents the basic unit of life, has become a desirable target for study given the inherent heterogeneous nature of biological systems and their associated diseases. The study of an individual element within a population allows information regarding the system's heterogeneity to be obtained. The small sample size requirement of single cell analysis is also advantageous in various fields of research from embryonic development studies to tumour biopsy analysis where the quantity of sample is limited. Typically in single cell studies the entire cell is consumed in the analysis. Using a miniaturized assay method, the goal of this research was to manipulate a single cell so that it can be repeatedly sampled. Multiple sampling permits single cell enzyme assays and enhances the diversity of the analysis. Individual cells were placed in 200 nL reaction vessels and 5 nL aliquots were removed using a recently developed nanopipettor. The efficient separation power of capillary electrophoresis coupled with the ultrasenstive detection capabilities of laser induced fluorescence were employed to analyse the reaction aliquots. The study of α-glucosidase II, a key enzyme in N-linked glycoprotein biosynthesis, is reported here. Individual Sf9 cells were incubated with the substrate αGlc(1→3)-αGlc which was labelled with a fluorescent tetramethylrhodamine tag. Twenty individual cells were analysed with respect to α-glucosidase II activity and they exhibited a high degree of heterogeneity. Utilizing the ability of continuous sampling, a single cell α-glucosidase II reaction profile was also obtained by removing several 5 nL aliquots over time. Further analyses, such as amino acid determination via NDA labelling, were employed to determine the nature of the observed heterogeneity in α-glucosidase II activity and to confirm the presence of one cell in each reaction vessel. The ability to perform multiple analyses on a single cell makes this method widely applicable and may offer insight into various cellular processes such as cell communication, differentiation, and metabolism.

(472) **Rapid Small-scale Isolation, Purification, and Quantification of Glycosaminoglycans.**

Daniel R. Studelska, Kari Giljum and Lijuan Zhang

Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110.

We present detailed methods for the rapid isolation of glycosaminoglycans (GAGs) from small tissue samples or cell cultures. Instead of columns, batch procedures that readily enable the parallel processing of samples are employed. We describe a sensitive and robust method of determining the molar concentrations of these GAGs. The method is based on a popular assay for amino acid analysis. We have refined it to enhance GAG quantification. It allows the separation and quantification of glucosamine- and galactosamine-containing GAGs after the reversed-phase separation of their fluorescent isoindole derivatives. The derivatives are created by the reaction of o-phthaldialdehyde and 3-mercaptopropionic acid with the amino group of hexosaminitol monosaccharides generated from GAG acid hydrolysis and sodium borohydride reduction. The advantages of our method include a simple chromatograph, clean separation of glucosaminitol and galactosaminitol derivatives from contaminating amino acids, excellent sensitivity, and linearity over a wide concentration range. It can be readily used in any laboratory with typical reversed-phase HPLC equipment.

(473) **Synthesis of α-cyclodextrin Linked Alginate: Application to the Nonylphenol Eradication**

Weeranuch Pluemsab, Tetsuya Furuike and Nobuo Sakairi
Graduate School of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan.

Since cyclodextrins (CDs) have a unique property to form inclusion complex, their applications in various fields have been extensively investigated. CDs are also regarded as useful materials for the eradication environmental pollutants such as endocrine disrupting chemicals that found the contamination in industrial effluents, sewage treatment plants and so on. Here we would like to report synthesis of a new immobilized CD, α-CD linked alginate and its property to form inclusion complex with nonylphenol. The coupling reaction between α-CD and alginate was designed at the hydroxyl groups of alginate matrixes in order not to affect the carboxyl groups, which are necessary to form calcium-alginate beads. We examined two different synthetic routes, part A and B. In part A, coupling α-CD was to perform after preparation of calcium-alginate beads. The hydroxyl groups of alginate beads were firstly activated by treatment with cyanogen bromide under alkaline conditions, and subsequently reacted with 6-amino-α-cyclodextrin (α-NH_2-CD). Although most of the resulting beads became breakage and alteration during the coupling process, its degree of substitution (D.S.) of α-CD was 0.16. Column chromatography showed that the α-CD-linked alginate beads had a property to strongly adsorb nonylphenol. An alternative approach to synthesize was to coupling α-NH2-CD at the sodium alginate polymer before forming the beads (part B). We examined with different weight ratios (CD to alginate) preparation. D.S. elucidated by ^{1}H-NMR spectroscopy of the modified polymer in weight ratio 2:1 and 5:1 was 0.05 and 0.25, respectively. In addition to polymeric alginate, we prepared fractions enriched in guluronic acid, mannuronic acid and the alternating sequence (G-fraction, M fraction, and MG-fraction, respectively) by partial acid hydrolysis of alginate, and evaluated the coupling reaction. The results indicated that all fractions had high D.S. value, which resulted from the low degree of polymerization of alginate and has higher terminal residues to provide free hydroxyl groups for the reaction. Since nonylphenol presents fluorescent property, it is interesting to analyze the changes produced in that property when it forms inclusion complex. The α-CD coupling with sodium alginate (weight ratio 5:1) was shown the significant enhancement in fluorescent spectra when formed an inclusion complex with nonylphenol while recorded with excitation and emission wavelength at 275 and 311, respectively. The circular dichroism spectra of α-CD linked alginate and nonylphenol complex also exhibited a significant change, with an increase in intensity region. This modified polymer could be still performed the beads in the presence of calcium ions, indicating that this approach might be performed without interfering the main reactive site of calcium-binding alginate. Under a Scanning electron microscope view, the modified polymer formed highly cross-linked alginate beads that mainly thickening at the surface of beads. This indicated that the method of part B is possibly available to use for bacterial encapsulation for nonlyphenol eradication from hazardous waste sites in the future purpose.

(474) **Puzzle in the Relationship between Glycosyltransferase Expression and Cell Surface *O*-glycans : A Structural Study with Human Colon Carcinoma Cells Transfected with β3Gal-T5 cDNA**

Daisuke Sugahara [1,2], Tatsuro Irimura [2], Hisashi Narimatsu [3] and Junko Amano[1]
[1] Laboratory of Glycobiology, The Noguchi Institute, [2] University of Tokyo, Graduate School of Pharmaceutical Sciences, [3] National Institute of Advanced Industrial Science and Technology .

Background : The structures of cellular glycans are generated by the expressions of glycosyltransferases. However, it is difficult to predict the glycosylation profiles and the range of heterogeneity of glycans on a cell, when a small alteration in the expression of glycosyltransferase occurs. The structural elucidation of all glycans produced by a cell is necessary, to understand the biosynthetic regulation and the biological significant of cellular glycans. However, structural elucidations of *O*-glycan have particularly been hampered for many reasons. The present work is designed to determine the alterations in the profile of *O*-glycans synthesized in human colon carcinoma cells before or after the expression of β1,3-galactosyltransferase 5 (β3Gal-T5) cDNA. We have previously established a quantitative oligosaccharide-labeling method using 1-pyrene butanoic acid hydrazide (1). In this work, this fluorescence-labeling method is applied to the structural elucidation of cellular *O*-glycans.
Methods : β3Gal-T5 is an enzyme to synthesize type 1 chain. HCT-15 cell, a human colon cancer cell line, is known not to express β3Gal-T5. *O*-glycans were prepared from stable transfectant cells of β3Gal-T5 cDNA and mock transfectant cells (2). Glycopeptides were obtained from delipidated cell pellets by Pronase treatment. The obtained glycopeptides were desalted using a centrifugal filter unit, Centricon YM-3 (Millipore) against 0.01% TFA and lyophilized. The cellular *O*-glycans were liberated by hydrazinolysis at 60□C for 5 hours. After re-acetylation, 1-pyrene butanoic acid hydrazide (Molecular Probes) was introduced into the reducing-end of oligosaccharide. The pyrene-labeled *O*-glycans were separated by HPLC equipped with a Cosmosil 5NH_2-MS column (Nacalai tesque, 4.6 x 150 mm). The glycan structures corresponding to each peak were elucidated by sequential digestions of glycosidases.
Results and Discussion : The predominant *O*-glycan derived from parental HCT-15 cells has Fucα1,2Galβ1,4GlcNAc- at non-reducing end. HCT-15 cells expressing β3Gal-T5 synthesized Galβ1,3 (Fucα1,4) GlcNAc- and NeuAcα2,3Galβ1,3 (Fucα1,4) GlcNAc- at non-reducing end. Although sialyl lewis X epitope was not detectable on parental HCT-15 cells, it was revealed that single transfection of β3Gal-T5 cDNA into HCT-15 cells resulted in the production of sialyl lewis a epitope. Interestingly, a conversion of type 2 chain (Galβ1,4GlcNAc-) to type 1 chain (Galβ1,3GlcNAc-), was associated with the alterations in fucosylation and sialylation of O-glycans. Cellular glycosylation could be entirely altered with the expression change of single glycosyltransferase.
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(475) **A Strategy for the Noninvasive Imaging of Glycosylation**

Jennifer A. Prescher[1], Danielle H. Dube[1] and Carolyn R. Bertozzi[1,2,3]
[1] Departments of Chemistry, [2] and Molecular and Cell Biology, University of California, Berkeley, and, [3] Howard Hughes Medical Institute.

A fundamental goal in the field of molecular imaging is the identification of tissue-specific markers that can be targeted with probes for image contrast. Aberrant glycosylation is a hallmark of malignancy and a feature of tumor cell surfaces that could, in principle, be exploited for targeted diagnostics. Numerous cancers have been shown to express elevated levels of oligosaccharide antigens bearing the monosaccharide sialic acid. Consequently, many cancer cells tend to express high levels of sialic acid compared to their normal counterparts. Therefore, an imaging strategy that targets sialic acid could potentially be used for tumor diagnostics. In previous work, we have shown that *unnatural* sialic acids can be introduced onto cells metabolically by feeding the cells analogs of their biosynthetic precursors. The unnatural analogs can be endowed with bioorthogonal functional groups capable of covalent reaction with exogenous probes. For example, an azide-functionalized analog of *N*-acetylmannosamine termed ManNAz is converted by cells to the corresponding sialic acid (SiaNAz) *in vivo*. The sialic acid-associated azides can be covalently tagged within living animals using triarylphosphine probes via the Staudinger ligation, enabling the delivery of reagents to cell surfaces that are rich in sialic acid. Here we present the development of a novel noninvasive imaging strategy that exploits azidosugars as metabolic markers for the covalent targeting of tumor cells with diagnostic agents. We synthesized an assortment of probes for three imaging modalities: radionuclide imaging, magnetic resonance imaging (MRI), and fluorescence imaging, and are currently exploring the utility of these reagents for glycan-specific imaging in murine tumor models. The ability to chemically modify cell surface glycans in living animals provides a means to monitor changes in glycosylation in a physiologically authentic context.

(476) **Identification of Important Amino Acid Residues in the Sialyl Motif S of α2,8-sialyltransferases**

Shou Takashima[1,2], Shuichi Tsuji[3] and Masafumi Tsujimoto[1]
[1] Laboratory of Cellular Biochemistry, RIKEN, Wako, Saitama, Japan, [2] (Present address) Glycochain Functions Laboratory, RIKEN, Wako, Saitama, Japan, [3] The Glycoscience Institute, Ochanomizu University, Otsuka, Bunkyo-ku, Tokyo, Japan.

ST8Sia I and VI are mammalian α2,8-sialyltransferases that are involved in the synthesis of ganglioside GD3 and disialic acid structures on *O*-glycans, respectively. The ST8Sia I and VI genes share a similar genomic structure, suggesting that they are evolutionally related to each other. However,

substrate specificities of these enzymes are different. To elucidate the important regions which are responsible for characterizing the enzymatic properties of these enzymes, we constructed chimeric enzymes of mouse ST8Sia I and VI, and assayed their enzymatic activity. Sialyl motifs (SM) L and S are highly conserved among mammalian sialyltransferases. Replacement of SM-L, which is involved in the binding of CMP-Sia, from ST8Sia I to ST8Sia VI and vice versa was almost no effect on their activity. But, most of other chimeric enzymes, such as having SM-S replacement, lost their activity. Then we introduced mutations in the SM-S of ST8Sia I and VI, as the SM-S is involved in the binding of acceptor substrates, and assayed their activity. The activity was lost by substitution of the highly conserved amino acid residues, such as Cys in the SM-S. However, the activity was also lost by substitution of some of the non-conserved amino acid residues (Gly 284 for ST8Sia I; Met 326, Gly 333, and Asn 337 for ST8Sia VI), suggesting that these amino acid residues may play important roles in characterizing the enzymatic properties of these enzymes.

(477) Sialooligosaccharides of α1-acid Glycoprotein in Some Mammalian Species

Miyako Nakano[1], Kazuaki Kakehi[1], Men-Hwei Tsai[2] and Yuan Chuan Lee[3]
[1] Faculty of Pharmaceutical Sciences, Kinki University, Kowakae 3-4-1, Higashi-Osaka 577-8502, Japan, [2] Ambryx Biotechnology Inc., 5603D Foxwood Drive, Oak Park, CA 91377, U.S.A., [3] Biology Department, Johns Hopkins University, North Charles Street, Baltimore, MD 21218, U.S.A..

α1-acid glycoprotein (AGP, orosomucoid), a highly heterogeneous glycoprotein having low pI value, is one of the major plasma proteins. AGP is produced mainly in liver, but extrahepatic synthesis has also been reported. AGP concentrations in normal sera are ca. 1 g/L in human and increase several-folds during acute-phase reactions, cancer, pregnancy and some other diseases. Marked changes in glycoforms (i.e. composition of oligosaccharides) have also been observed.

Carbohydrate chains of AGP samples from human, bovine, sheep, and rat were released with glycoamidase F and labeled with 2-amino benzoic acid (2-AA). The fluorescent-labeled sialooligosaccharides were well resolved based on the number of sialic acids, and isomers of oligosaccharides having sialic acids at different positions using HPLC with a polymer-based amino stationary phase, and each peak being further analysed by MALDI-TOF MS.

Human AGP contains di-, tri- and tetra-antennary glycans, and some of the tri- and tetra-antennary carbohydrate chains are substituted with a fucose (sialyl Lewis X type structure). Sheep AGP contains mono- and disialo-diantennary glycans as major oligosaccharides. Tri- and tetra-sialo triantennary carbohydrate chains are also present as minor oligosaccharides, and some of the sialic acid residues are *N*-glycolylneuraminic acid. Rat AGP contains quite complex mixtures of disialo carbohydrate chains having *N*- and *O*-acetyl groups in sialicd acid residues. Triantennary carbohydrate chains carrying *N*- and *O*-acetylneuraminic acids are also observed. We found some novel carbohydrate chains containing both *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid in bovine AGP. Interestingly, triantennary carbohydrate chains are hardly detected in bovine AGP but diantennary carbohydrate chains with tri- or tetra-sialyl residues are abundantly present. Furthermore, major sialic acid in these glycans is *N*-glycolylneuraminic acid.

We show that capillary affinity electrophoresis (CAE) is a powerful tool for analyzing the sialic acid moieties of bovine AGP oligosaccharides, and interactions between sialic acid binding lectins (SSA for α2-6 and MAM for α2-3 sialic acid residues, respectively) and the carbohydrate chains labeled with 2-AA. Based on the lectin specificities and additional chemical analyses, we found that sialic acids in bovine AGP are attached to multiple sites of the glycans, and are not present as Sia-Sia groups.

(478) Site-specific N-glycosylation Analysis of Human Apolipoprotein B100 using LC/ESI/MS/MS

Akira Harazono, Nana Kawasaki, Toru Kawanishi and Takao Hayakawa
1-18-1 Kami-yoga, Setagaya-Ku, Tokyo 158-8501, Japan.

Apolipoprotein B100 (apoB100) is the only protein component of low-density lipoprotein. Human apoB100 consists of 4536 amino acids and its molecular weight has been calculated to be 513 kd. ApoB100 has 19 potential N-glycosylation sites, and 16 asparagine residues were reported to be occupied by high-mannose type, hybrid type, and monoantennary and biantennary complex type oligosaccharides. In the present study, a site-specific glycosylation analysis of apoB100 was carried out using reversed-phase high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS). Reduced and carboxymethylated apoB100 was digested by trypsin or chymotrypsin. The complex mixture of peptides and glycopeptides was subjected to LC/ESI/MS/MS, where product ion spectra of the molecular ions were acquired data-dependently. The glycopeptide ions were extracted and confirmed by the presence of carbohydrate-specific fragment ions such as *m/z* 204 (HexNAc) and 366 (HexHexNAc) in the product ion spectra. The peptide moiety of glycopeptide was identified by the presence of the b- and y-series ions derived from the peptide in the product ion spectrum, and the oligosaccharide was deduced from the calculated molecular mass of the oligosaccharide. Our data from tryptic digest and chymotryptic digest showed that Asn2212, not previously identified as a glycosylation site, could be glycosylated and revealed the heterogeneity of carbohydrate structures at 17 glycosylation sites. It was revealed that Asn158, 1341, 1350, 3309, and 3331 were occupied by high-mannose type oligosaccharides while Asn 956, 1496, 2212, 2752, 2955, 3074, 3197, 3438, 3868, 4210 and 4404 were predominantly occupied by mono- or disialylated biantennary complex type oligosaccharides. Asn 3384, the nearest N-glycosylation site to the LDL-receptor binding site (amino acids 3359-3369), was occupied by a variety of oligosaccharides including high-mannose, hybrid and complex types.

(479) PIG-V Transfers the Second Mannose to Glycosylphosphatidylinositol.

Ji Young Kang[1], Yeongjin Hong[2], Nobue Shishioh[1], Hisashi Ashida[1], Yusuke Maeda[1] and Taroh Kinoshita[1]
[1] Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. , [2] Genomic National Center for Enteropathogenic Bacteria and Department of Microbiology, Chonnam National University Medical School, Gwangju, S.Korea..

Glycosylphosphatidylinositol (GPI) is a glycolipid that anchors many proteins to the eukaryotic cell surface. Biosynthetic pathway of GPI is mediated by the sequential additions of sugars and other components to phosphatidylinositol (PI). Four mannoses in the GPI are transferred from dolichol-phosphate-mannose (Dol-P -Man) and are linked through different glycosidic linkages. Therefore, four Dol-P-Man-depedent mannosyltranferases, GPI-MT-I, -MT-II, -MT-III and -MT-IV for the first, second, third and fourth mannoses, respectively, are required for generation of the GPI. GPI-MT-I (PIG-M), GPI-MT-III (PIG-B) and GPI-MT-IV(SMP3) were previously reported but GPI-MT-II has not been identified. Recently, we obtained new mutant cells defective in the surface expression of GPI anchored proteins from chemically mutagenized CHO cells. We termed these mutant cells class V. We demonstrated that class V cells accumulate two GPI intermediates H2 (Man-GlcN-acyl-PI) and H5 (EtNP-Man-GlcN-acyl-PI) by metabolic labeling with [3H]Man. Since both intermediates did not contain the second Man, the class V mutants may be defective in the second mannosyltrasferase GPI-MT-II. By expression cloning, we obtained a gene responsible is termed PIG-V, which encodes 493 amino acids containing eight putative transmembrane regions. To further characterize the function of PIG-V, we performed homology search and found that one ORF of Saccharomyces cerevisiae has 25% amino acid identity to human PIG-V. Human PIG-V restored viability of a yeast PIG-V deletion mutant, suggesting that they are functional homologues. The PIG-V homologues share two highly conserved regions. To determine the membrane topology of these regions, we transfected human PIG-V tagged with GST at the N-terminus in CHO cells and showed N-terminus is facing cytoplasm using immunofluoresencent microscopy. Because the first conserved region is located between the first and second transmembrane regions, we presumed that the first conserved region is facing ER lumen. To determine the membrane topology of the second conserved region, we introduced artificial N-glycosylation sites into this region, and found that these sites were N-glycosylated, suggesting that this region also faces ER lumen. Because the active site of the enzyme is expected to face the ER lumen, we considered the possibility that amino acid residues in these conserved regions are involved in the catalysis. To test this hypothesis, we introduced a number of point mutations into these two regions and found point mutation of 4 conserved amino acid residues resulted in a lack of the surface expression of GPI-anchored proteins. In particular, we found based on sequence aliment that are aspartic acid in PIG-V corresponds to an aspartic acid residue that is thought to be involved in stabilizing metal ion during catalysis in many other glycosyltransferases, and that aspartic residue in PIG-V idea was essential for the function of PIG-V. These data are consistent with the possibility that these conserved regions form a

catalytic site. Taken together, we suggest that PIG-V is the second mannosyltransferase in GPI anchor biosynthesis.

(480) **Preparation and Estimation of Affinity Membrane with Immobilized Glycoconjugate Polymers**

Atsushi Miyagawa, Maria Carmelita Kasuya and Kenichi Hatanaka
Institute of Industrial Science, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan.

Cellulose could be easily modified and utilized as filter paper or as dialysis membrane. Furthermore, modified cellulose derivative was utilized as ion-exchange adsorbents and as separation membranes. Glycoconjugate polymers have specific adsorbability for biomolecules such as virus, toxin, lectin, and carbohydrate. In this research, glycoconjugate polymers were immobilized to cellulose membrane. The cellulose membranes with immobilized glycoconjugate polymers can be utilized for affinity membrane chromatography.

Two kinds of glycoconjugate polymers, having lactose or mannose, were synthesized from different glycosylation procedures. Initially, the glycoconjugate polymer having lactose was synthesized as follows: Octaacetyl b-lactose was glycosylated with 5-hexen-1-ol using BF_3•Et_2O and then deacetylated to afford 5-hexenyl b-lactoside. A solution containing 5-hexenyl b-lactoside and aminoethanthiol hydrochloride were irradiated (254 nm) and gave an amine hydrochloride derivative. The amino group at w-position of the aglycon was *N*-acryloylated and subsequently acetylated for purification. Deacetylation gave the lactosyl monomer having an acrylamide group at the reducing end. Next, the glycoconjugate polymer having mannose was synthesized as follows: Pentaacetyl mannose was 1-*O*-deacetylated by benzylamine and activated by $SOCl_2$ to afford tetraacetyl a-mannosyl chloride. Then mannosyl chloride was glycosylated with 5-hexen-1-ol using silver trifluoromethansulfonate as promoter to afford 5-hexenyl acetyl a-mannoside which was deacetylated to give 5-hexenyl a-mannoside. 5-Hexenyl a-mannoside was converted to a mannosyl monomer having an acrylamide group at the reducing end by the same procedure as the lactose monomer *via* radical addition, *N*-acryloylation and deacetylation. Finally, hexamethylenediamine was mono-*N*-acryloylated via simple procedure to afford an amine monomer that will serve as immobilization point. These monomers were copolymerized at room temperature using *N*, *N*, *NÅå*, *NÅå*-tetramethylenediamine and ammonium persulfate as initiators. The copolymerized ratio of glycosyl monomer (lactose or mannose) and amine monomer was twenty to one. The synthesized glycoconjugate polymers were immobilized to cellulose membranes.

Cellulose membranes were carboxymethylated (CM) to afford CM-cellulose membranes. The CM-cellulose membranes were used as scaffold to immobilize glycoconjugate polymers. The glycoconjugate polymers (having lactose or mannose) and amine were immobilized to the CM-cellulose membranes by condensation. Consequently, the adsorbability for lectins of the glycoconjugate polymers immobilized to the cellulose membranes was estimated. The cellulose membranes (5 pieces) were set in a filter holder to which a solution containing lectin (200ug/mL, 1mLÅ~2) was flowed to adsorb to the membranes. Then the membranes were washed with PBS, and eluted using 0.2 M mannose-PBS or 0.2M lactose-PBS solution. The amount of lectin was estimated from all fractions collected. In this experiment, concanavalin A (ConA) and *Ricinus communis agglutinin* (RCA120) were applied. ConA is a mannose-binding lectin while RCA120 is a galactose binding-lectin. The results showed that the cellulose membranes adsorbed about 50% and 80% of ConA and RCA120, respectively.

This research showed that cellulose membranes with immobilized glycoconjugate polymers could be utilized for affinity membrane chromatography. Moreover, not only lectins but also various proteins could also be adsorbed by changing and modifying the immobilized polymers.

(481) **Development of Lectin-microarrays to Profile Glycoprotein Glycosylation under Equilibrium Conditions with an Evanescent Field Fluorescenceå@detection Principle.**

Noboru Uchiyama[1], Youji Ebe[2], Masao Yamada[2], Atsushi Kuno[1] and Jun Hirabayashi[1]
[1] Research Center for Glycoscience (RCG), National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki, Japan, [2] Moritex Corporation, Shibuya, Tokyo, Japan.

Development of carbohydrate biosensors including microarrays has recently attracted increased attention to investigate carbohydrate-lectin interactions in a comprehensive and high-throughput manner. Carbohydrate microarrays are emerging techniques to meet such requisites. However, most of these methods adopt a conventional immuno-detection system, which requires repetitive washing steps before detection. Since most of interactions between lectins and carbohydrate are weak compared with those between antigens and antibodies, a more precise analytical method, which does not require a washing step, is desired. Here, we developed a lectin microarray platform for direct observation of lectin-carbohydrate interaction under equilibrium conditions on the basis of an evanescent field fluorescence detection principle. To validate the developed system, we printed 5 spots each for 40 commercially available plant lectins on a slide glass, which has 4 wells, and probed with four different Cy3-labeled glycoproteins in parallel under equilibrium conditions. The bound glycoproteins were directly detected with no washing procedure by using an evanescent field fluorescence scanner. As a result, profiles of bound fluorescence spots were obtained for the different glycoprotein probes, which clearly reflected the established oligosaccharide specificities of the used lectins. It was confirmed that lectin-glycoprotein interactions were specifically abolished in the presence of potent saccharide inhibitors. This method allowed us to analyze a panel of glycoproteins in an extremely sensitive manner. It required a glycoprotein sample solution of less than 200 É L at a concentration of 100 ng/mL or less to reproduce multiple lectin profiling. The system also allowed real-time observation of lectin-glycoprotein interactions to differentiate association reactions among different lectins. Thus developed system is expected to be useful for various fields of glycomics requiring high-throughput analysis of glycoproteins. Further studies using oligosaccharide probes are also in progress. This work is partly supported by the New Energy and Industrial Technology Development Organization (NEDO) in Japan.

(482) **Comprehensive Interaction Analysis between Lectins and PA-oligosaccharides by an Automated Frontal Affinity Chromatography (FAC) System**

Sachiko Nakamura[1], Noboru Uchiyama[1], Atsushi Kuno[1], Junko Kominami[2], Nobuyuki Tatsumi[3], Yusuke Osaka[3], Shuzo Maruyama[3], Teruhisa Ueda [3] and Jun Hirabayashi[1]
[1] Research Center for Glycoscience, AIST, Tsukuba Central 6, 1-1-1, Higashi, Tsukuba, Ibaraki, 305-8566, Japan, [2] J-OIL MILLS, 11, Kagetori, Totsuka, Yokohama, 245-0064, Japan, [3] SHIMADZU Corp., 1, Nishinokyo-Kuwabara, Nakagyo, Kyoto, 604-8511, Japan.

Importance of protein glycosylation in physiological phenomena has been emphasized. However, both structural complexity due to isomerism and branching and poor availability of functionally important glycans make it extremely difficult to analyze their structures rapidly. To overcome these problems, utilization of glycan-binding proteins (e.g., lectins) to profile glycans is promising, considering the facts that in general lectins have more diverse specificities showing a much wider range of binding affinity (e.g., 10^{-3} M to 10^{-8} M in dissociation constant, Kd) than antibodies. For this realization, accumulation of a fundamental database on lectin-oligosaccharide interactions is necessary. To achieve this purpose, we developed an automated frontal affinity chromatography (FAC) system, which can provide us with precise information on Kd using a robust system in a high-throughput manner. With this system, "Hect-by-Hect project", which intends to construct the "Lectin Map" for interaction between 100 lectins and 100 glycans, has started. To begin with, interaction between 49 oligosaccharides to represent glycan epitopes, including 16 N-linked glycans and 28 glycolipids-type glycans, and 42 lectins from various sources, that is, plant, fungi, bacteria and animals, were analyzed. As a result, the first-step "Lectin map" was constructed, from which we could easily identify epitope structures specifically recognized by individual lectins. Importantly, "Lectin Map" also tells us that various glycan structures can be discriminated from one another by comparing the patterns of multiple lectin affinities. In this context, the constructed FAC system proved to be a powerful tool to accumulate reliable data on lectin-oligosaccharide interactions, and thus, the "Lectin Map" should contribute to developing a glycan profiler. Detailed analysis using 103 glycans is in progress.

This work is supported in part by the New Energy and Industrial Technology Development Organization (NEDO) in Japan.

(483) **Glycomics Analyses: Automatic Annotation of Maldi Spectra**

David Goldberg[1], Simon J. North[2], Mark Sutton-Smith[2], Stuart M. Haslam[2], James Paulson[3], Howard R. Morris[2,4] and Anne Dell[2]
[1] Scripps-PARC Institute for Advanced Biomedical Sciences, 3333 Coyote Hill Road, Palo Alto, CA, USA, [2] Department of Biological Sciences, Imperial College London, SW7 2AZ, UK, [3] Departments of Molecular

Biology and Molecular Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, MEM-L71, La Jolla, CA 92037 , [4] 4M-Scan Mass Spectrometry Research and Training Centre, Silwood Park, Ascot, SL5 7PZ, UK.

MALDI-MS is the pre-eminent technique for mass mapping of glycans. In order to make this technique practical for high-throughput screening, reliable automatic methods of annotating peaks must be devised. We describe an algorithm called Cartoonist, that labels peaks in MALDI spectra of permethylated N-glycans with cartoons which represent the most plausible glycans consistent with the peak masses and the types of glycans being analysed. There are three main parts to Cartoonist. First it selects annotations from a library of biosynthetically plausible cartoons. The library we currently use has about 2800 cartoons, but was constructed using only about 300 archetype cartoons entered by hand. Second it determines the precision and calibration of the machine used to generate the spectrum. It does this automatically based on the spectrum itself. Third it assigns a confidence score to each annotation. In particular, rather than making a binary yes/no decision when annotating a peak, it makes all plausible annotations and associates them with scores indicating the probability that they are correct.

Cartoonist is now being used on a trial basis to label data[1] from the Analytical Core of the NIH Consortium for Functional Glycomics[2], running in parallel with the manual annotation process. We report here the preliminary results of the comparison between the two methods and provide a practical overview for use of Cartoonist.

[1] acquired by M-Scan Inc, West Chester, PA

[2] http://www.functionalglycomics.org

(484) **Construction of Oligosaccharide and Glycopeptide Libraries using a Human Glycosyltransferase Library.**

Hiromi Ito[1], Akihiko Kameyama[1], Takashi Sato[1], Katsue Kiyohara[1], Yoshiaki Nakahara[2] and Hisashi Narimatsu[1]

[1] Glycogene function Team, Research Center for Glycoscience(RCG), National Institute of Advanced Industrial Science and Technology(AIST), [2] Department of Applied Biochemistry, Institute of Glycotechnology, Tokai University.

Libraries of oligosaccharides and glycopeptides are eagerly awaited to be constructed for many purposes. In general, there are two major methods for the synthesis of oligosaccharides, i.e. chemical or enzymatical synthesis. The major advantages of enzymatic syntheses are as follows. First, glycosidic bonds are formed a strict specificity for substrates of glycosyltransferases. Second, the variety of oligosaccharide structures in an organism, which is strictly controlled by glycogenes, is much smaller than that expected by mathematical calculation. Thus, it is easier to construct a physiological library by the natural enzymes than by the chemical synthesis. We have already cloned many human glycosyltransferases, and it is possible for systematic preparation of oligosaccharide and glycopeptide libraries. For the synthesis of O-glycan peptide, the basic structure was synthesized chemically. Extension of O-glycans was achieved by sequential addition of each enzyme in a single tube. Each reaction was stopped at around a 50% yield by monitoring the time course of the reaction using MS, and the next enzyme and the donor substrate were added to the mixture. Thus, we are now constructing a glycopeptide library containing various O-glycan structures as many as possible. This work was supported by New Energy and Industrial Technology Development Organization (NEDO).

(485) **Structural Characterization of Glycopeptides by N-terminal Protein Ladder Sequencing**

Yusuke Suzuki[1], Yoshiaki Nakahara[2], Yukishige Ito[3], Kozo Miseki[4], Minoru Suzuki[1] and Akemi Suzuki[1]

[1] Sphingolipid Expression Lab., RIKEN Frontier, [2] Dept. of Synthetic Cellular Chem., Tokai University, [3] Dept. of Cellular Biochem., RIKEN, [4] Analytical Div., Shimadzu Corp.

Glycoproteins are an important group of biomolecules and comprise about half of all proteins from eukaryotic sources. The characterization of protein glycoforms is desirable because glycosylation affects biological activity, stability and solubility of the proteins. Because of difficulties in structural characterization with a small amount of intact glycoproteins, glycoproteins can be converted to glycopeptides by enzymatic digestion and these glycopeptides can be subjected to the structural characterization by mass spectrometry, which is a best tool at present for the glycomics analysis. Synthetic glycopeptides were analyzed by matrix- assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry (MALDI-QIT-TOF MS), which enables CID-MS^n analysis for fine structural characterization. The CID-MS/MS of the protonated molecular ions provided information on sugar sequences of oligosaccharide moieties and amino acid sequences of the glycopeptides. The CID-MS/MS of the alkaline metal adduct ions selected as the first precursor ions gave additional information on the attached sites of the oligosaccharides. These spectra were still complex and especially it is difficult to acquire the information about the attached sites of the oligosaccharides. Therefore, in order to obtain simpler and clearer spectra than CID-MS/MS spectra, we applied N-terminal protein ladder sequencing (Chait et al., Science. 1993) which combines multiple steps of wet Edman degradation and a single-step MALDI-TOF MS. The spectra obtained by this method with chemically synthesized glycopeptides were simple and clear and provided information on not only sugar and amino acid sequences but also attached sites of oligosaccharides.

(486) **Approaches for Characterizing ppGalNAc Transferase Peptide Substrate Specificity**

Thomas A. Gerken and Jason Rarick

Case Westren Reserve Univ., Deptartments of Pediatrics and Biochemistry, School of Medicine BRB, 2109 Adelbert Rd, Cleveland, OH 44106.

A large family (>20) of UDP-α-N-acetylglactosamine: polypeptide N-acetylgalactosaminyl transferases (ppGalNAc T's) initiate mucin-type O-glycan biosynthesis by transferring α-GalNAc to peptide Ser and Thr residues. Homologous family members are found across a wide range of species from *Caenorhabditis elegans* to man. In *Drosophila* one ppGalNAc T homologue is required for development, suggesting that several ppGalNAc T's may have unique substrate specificities, evolutionarily maintained for the efficient glycosylation of specific peptide sequences (Schwientek et. al., J. Biol. Chem. *277*, 22623 (2002), Ten Hagen & Tran, J. Biol. Chem. *277*, 22616 (2002)). Presently, the peptide substrate specificities of the individual ppGalNAc T family members are not well characterized or understood, leading to an inability to rationally predict or comprehend transferase specific O-glycosylation. To address this deficiency we have utilized large apo-mucin tandem repeats containing multiple Ser and Thr residues as ppGalNAc T substrates. Combined, these substrates represent 50+ unique glycosylation sites. Using Edman amino acid sequencing the site specific glycosylation patterns of the tandem repeats for both ppGalNAc T1 and T2 have been determined. By characterizing the rates of site specific glycosylation, in terms of neighboring glycosylation and peptide sequence, we have developed a kinetic modeling approach capable of approximating the observed ppGalNAc T1 and T2 glycosylation patterns (Gerken et. al., J. Biol. Chem. *277*, 49850 (2002), Gerken et. al., Biochemistry *43*, 9888 (2004)). This approach has identified a ppGalNAc T2 motif and is capable of reproducing the ppGalNAc T2 glycosylation patterns for the IgA1 hinge domain. The use of apo-mucin tandem repeat substrates represents a laborious and less than thorough approach for obtaining ppGalNAc T peptide sequence specificity. To further explore transferase specificity we have designed a semi-random peptide substrate containing a central Thr residue flanked on both sides by a triad of randomized residues (~XXXTXXX~, where X= G,A,P,N,E,R,Y & V). Preliminary experiments utilizing this substrate for both ppGalNAc T1 and T2 have been performed. Edman sequence analysis of the glycosylated substrate (isolated by immobilized lectin column chromatography) revealed sites of specific amino acid residue enrichment (or loss) relative to the initial random peptide distribution. Our initial findings suggest the transferases prefer different neighboring residues: Pro, Val & Tyr for ppGalNAc T1, and Pro, Gly & Ala for ppGalNAc T2. Furthermore, the Pro residue enhancements appear to be consistent with the Pro weighting parameters derived previously from the apo-mucin kinetic modeling. With further optimization, we anticipate that the use of the random peptide substrate will provide additional residue specific weighting parameters for inclusion in our kinetic model. A scheme for characterizing ppGalNAc transferases with strong glycopeptide activities has also been devised. We anticipate that the continued characterization of ppGalNAc transferase specificity by the above approaches will lead to the identification of isoform-specific substrates, the creation of isoform-specific inhibitors and the prediction of mucin-type O-glycosylation sites. (Supported by the National Institutes of Health, grant RO1-CA-78834).

(487) **Capillary Affinity Electrophoresis for the Analysis of Carbohydrates Derived from Milk and Glycosphingolipids**

Kazuki Nakajima[1,3], Tadasu Urashima[2], Minoru Suzuki[3], Akemi Suzuki[3] and Kazuaki Kakehi[1]

[1] Faculty of Pharmaceutical Sciences, Kinki University, [2] Dept. of Life

Science & Agriculture, Obihiro University of Agriculture & Veterinary Medicine, [3] Sphingolipid Expression Lab., Frontier, RIKEN.

We developed capillary affinity electrophoresis (CAE) to analyze the molecular interaction between a mixture of carbohydrate chains and proteins in a solution (Nakajima *et al*., 2003). In the previous paper, we applied CAE to detect lectins in the crude extract derived from Tulip bulbs, and determined fine carbohydrate-binding specificity and affinity constant (Ka) of a *Tulipa gesneriana* agglutinin (TGA) (Nakajima *et al*., 2004). CAE can also characterize each carbohydrate in a complex mixture using lectins whose binding specificities are well established with high resolution and sensitivity when carbohydrates are labeled with fluorescent tags such as 8-aminopyrene-1,3,6-trisulfonate (APTS) and 3-aminobenzoic acid (3-AA). In this study, we applied CAE to characterize oligosaccharides derived from milk and glycosphingolipids. We selected an appropriate set of lectins which bind particular carbohydrates in a specific manner and cover a wide range of oligosaccharide structures. The method could classify not only core types of carbohydrates but also linkage isomers. For example, various fucose residues linked through α(1-2), α(1-3), α(1-4), or α(1-6) were efficiently classified by using *Ulex europaeus* agglutinin I (UEA-I), *Aleuria aurantia* lectin (AAL), *Aspergillus oryzae* lectin (AOL), and *Rhizopus stolonifer* lectin (RSL). Positional isomers of sialic acid, α(2-3) and α(2-6), were clearly differentiated by two sialic acid-binding lectins. Using these methods, we identified two interesting isomers of di-fucosylated milk oligosaccharides as follow: Galα(1-3)[Fucα(1-2)]Galβ(1-4)[Fucα(1-3)]GlcNAcβ(1-3)Galβ(1-4)Glc, and Galα(1-3)Galβ(1-4)[Fucα(1-3)]GlcNAcβ(1-3)Galβ(1-4)[Fucα(1-3)]Glc. Based on the data thus compiled, we are constructing a Web-based database to utilize the CAE more effectively. This database will enable us to predict characteristic structures of carbohydrates in biological samples by matching the result of the affinity of carbohydrates with selected lectins in high-throughput.

(488) **A High Throughput Erythropoietin Competition Binding Assay for Determining Relative Affinities of Glycopegylated Erythropoietin and Detecting Neutralizing Antibodies from Serum Samples.**

Eric R. Sjoberg[1], Dominique Gouty[1], Gloria Machado[1], Shawn Defrees[2] and David Zopf[2]

[1] 6330 Nancy Ridge Dr. San Diego, CA 92121, [2] 102 Rock Rd. Horsham PA 19044.

We are developing a form of extended half-life erythropoietin (EPO) in which polyethylene glycol (PEG) is covalently linked to sialic acid at one or more termini of N-glycan chains. To rapidly compare affinities of EPO constructs containing different numbers and sizes of attached PEG molecules, we have developed an ELISA based competition binding assay that employs the extracellular domain of the human EPO receptor fused to a mouse IgG2b Fc region. The recombinant soluble chimeric receptor,,when bound to immobilized recombinant Protein-A or -G, is immobilized in correct orientation to bind Europium labeled native erythropoietin. Competition binding curves are generated by estimating bound versus free [Eu]EPO in the presence of a range of concentrations of glycoPEGylated EPO. Affinities of glycoPEGylated EPO constructs tend to vary inversely with the number and size of PEG substituents. The assay gives Ki values for commercially available EPO standards that closely approximate published values. The use of time resolved fluorescence to detect Eu-labeled protein confers excellent sensitivity and avoids the need for radioactivity. This assay also has been configured to serve as a high throughput pre-screen to detect neutralizing anti-EPO antibodies in pre-clinical serum samples, a much simpler approach than previously used in vitro cellular proliferation assays.

(489) **An Immunogenic Non-Human Sialic Acid In Human ES Cells**

Maria J Martin[1], Alysson Muotri[2], Fred Gage[2] and Ajit Varki[1]

[1] Glycobiology Research and Training Center and Department of Medicine and Cellular and Molecular Medicine, University of California, San Diego.La Jolla, CA, USA, [2] The Salk Institute for Biological Studies. La Jolla, CA, USA.

The potential of human embryonic stem cells (HESC) to differentiate into any cell type or tissue makes them excellent candidates for therapy of many diseases. To be safe for transplantation, HESC-derived cells and tissues must be free of xenogenic components that could trigger an immune response in the patient. Almost all currently existing HESC cell lines are poorly suited for human treatment, since have been in contact with non-human cells (a mouse feeder layer) and/or animal products (serum or 'serum replacements'). These xenogenic culture conditions not only increase the risk of infection by non-human pathogens but also, as we address here, the possibility for HESC to incorporate the immunogenic non-human sialic acid N-glycolylneuraminic acid (Neu5Gc). We used flow cytometry to detect Neu5Gc on surfaces of HESC growing under such standard culture conditions and confirmed its presence by chemical analysis, showing that it represented up to 10% of the total sialic acids in HESC and up to 17% in embryoid bodies (EB), the first step in HESC differentiation, despite the latter grow in the absence of the feeder layer. While the HESC could incorporate some Neu5Gc from the feeder layer, the major source for both HESC and EB appears to be the serum-replacement (129 nmoles/mL of Neu5Gc). Sialic acids seem to be important for both undifferentiated HESC and also the in vitro derived cells. Many HESC markers are sialylated glycoproteins (such as TRA-1-60) and glycolipids (SSEA-4 is sialylGb5) and neuron-like cells derived from HESC express polysialylated NCAM and A2B5 gangliosides. Thus, the potential effect of this foreign Neu5Gc on HESC biology needs to be explored. Regarding transplantation, it is known that many healthy humans have circulating anti-Neu5Gc antibodies. We asked if these 'natural' antibodies could recognize the HESC growing under the standard conditions and if this binding caused activation of complement, which could eventually lead to the killing of any HESC-derived transplant. Human IgG and complement factor C3b were indeed detected on HESC cell surfaces after exposure to human sera with high level of anti-Neu5Gc antibodies and this was much less after exposure to human sera with low levels of such antibodies. To reduce the Neu5Gc content of HESC and EB, we substituted the serum-replacement with heat inactivated type AB pooled human serum with very low level of natural anti-Neu5Gc antibodies. HESC were able to maintain an undifferentiated state when cultured in such conditions, without losing the ability to differentiate into EB. After a week in the new medium, the percentage of Neu5Gc dropped to 1.2% in the HESC and to 0.2% in the EB. Moreover, this process markedly reduced the IgG and C3b deposition caused by exposure to human sera with high levels of anti-Neu5Gc antibodies. Thus, the metabolic uptake and incorporation of Neu5Gc by HESC growing under the currently accepted culture conditions could trigger an immune response. This danger could be markedly reduced by a short-term incubation in heat inactivated human serum from the transplant recipient.

(490) ***De novo* MS^n Sequencing with Contiguous Glycan Segments (CGS)**

Vernon Reinhold, Suddham Singh, Hailong Zhang and Andy Hanneman

Department of Chemistry, Biochemistry and Molecular Biology University of New Hampshire, Durham, New Hampshire 03824.

Ion trap disassembly (MS^n) of methylated glycans provides multiple pathways to evaluate detailed glycan structure. Methylation fixes the oligomer array and MS^n bond rupture in CID exposes positions of former connectivity. We define this point of previous association as a scar which alters a fragment's mass. As an example, simple glycosidic cleavage of a disaccharide produces a non-reducing terminal fragment with one scar, (unsaturation), and a reducing-end fragment with a different scar type (exposed hydroxyl). In the same manner an internal disaccharide would show two scars, and if a branched internal disaccharide, three, etc. A cross-ring cleavage also contributes to a mass shift and represents a scar of a different kind, with mass shifts that vary with the linkage position. Thus, an MS^n spectrum is simply a representation of monomer intervals with adjacent satellite peaks cataloging different scar types and their number in the form of mass shifts. From this uncomplicated assumption, we have generated an *in silico* ion fragment composition library (IFCL) for common eukaryotic carbohydrate monomers in which all scars are imagined. An actual library, *in natura*, is being compiled from known standards and previously characterized glycans. This *in natura* library is limited to actual scars of association, and additional fragments that characterize stereochemistry in product spectra that arise from facile fragmentation to dissipate resonance excitation and collision energies. These segments of structure are ordered into sequential arrays by MS^n disassembly which may be akin to a hierarchal tree progression from trunk to leaves with the IFCL defining all possible points of association. Importantly, sequential disassembly pathways in combination with the IFCL define Contiguous Glycan Segments (CGS). Any alteration from structural continuity defines isobars. Selected ion segments have been studied extensively by MS^n, and are proceeding with synthesis, and modeling to provide a structural relationship for fragmentation, monomer and anomer identification.

Experiments were carried out using a MALDI-IT (Kratos-Shamadzu), LCQ, and LTQ (Thermo) mass spectrometers. Supported by BRIN-NCRR(VR) and NIGMS(VR).

(491) **Glycan Characterization using a MS^n Fragment Fingerprint Library**

Hailong Zhang, Suddham Singh and Vernon Reinhold
Center for Structural Biology, Department of Chemistry University of New Hampshire, Durham, NH 03824.

Mass spectral library searching is an effective method to rapidly derive structural information from mass spectra. We have previously shown that spectra of common monomers, small oligomers, and their substructures can provide 'fingerprints' of glycan structure to reveal inter-residue linkage, monomer identification, anomeric configuration, and branching. Furthermore, the reproducibility of such spectra from different instruments, establishes the basis for library building. In this report we outline progress building a glycan fragment fingerprint (GFF) library derived from synthesized and well characterized methylated standards. Spectral authenticity and database entry is accepted when identity is shown from two different original sources. To date, the spectral fingerprint library has been successful in identifying fine structural details for various glycomer samples from human milk, chicken ovalbumin, *C. elegans*, and porcine submaxillary mucin. Experiments were carried out using a MALDI-IT (Kratos-Shamadzu), LCQ, and LTQ (Thermo) mass spectrometers. Supported by BRIN-NCRR (VR) and NIGMS (VR).

(492) **Stem Sequence at the N-terminal Region of B-1,4-Galactosyltransferase T5 and T6 Increases the Folding Efficiencies of their Respective Catalytic Domains by Step-Wise or Single Step Folding Method**

Himanshu Dubey[1], Boopathy Ramakrishnan[2], Elizabeth Boeggeman[2] and Pradman K. Qasba[1]
[1] Laboratory of Experimental and Computational Biology, Center for Cancer Research, NCI-Frederick, National Institutes of Health, Frederick, Maryland 21702, [2] Structural Glycobiology Section and d Intramural Research Support Program-SAIC, Laboratory of Experimental and Computational Biology, Center for Cancer Research, NCI-Frederick, National Institutes of Health, Frederick, Maryland 21702.

Glycosyltransferases constitute a super family of enzymes that are involved in the synthesis of complex carbohydrates present on the glycoproteins and glycolipids. The enzymes of the super family accomplish this task by engaging in the transfer of glycosyl moiety from the nucleotide derivatives to various sugar acceptors. b-1,4-Galactosyltransferase (b4Gal-T) is one of the subfamilies of the galactosyltransferase family that is constituted of at least seven members, bGal-T1 to bGal-T7. b4Gal-T5 has been implicated to have a role in O-glycosylation, while b4Gal-T6 is implicated as a lactosylceramide synthase, important for glycolipid biosynthesis. b4Gal-T5 and b4Gal-T6 proteins were expressed in E. coli with and without the stem portion (ST) at the N-terminal region of the catalytic domain (CD). Like many recombinant proteins b4Gal-T5 and b4Gal-T6 also aggregate and accumulate in E. coli as inclusion bodies. Previously we have shown that stem region enhances the in-vitro folding efficiency of the catalytic domain of both human and bovine b4Gal-T1. Catalytic domains (CD) of b4Gal-T5 and b4Gal-T6 fused to their corresponding stem region (ST)- the STCD-b4Gal-T5 and STCD-b4Gal-T6- were subsequently folded in-vitro from the inclusion bodies in a single-step dilution method, in the presence and absence of PEG-4000 and Arginine (PEG/Arg). The presence of PEG/Arg during in-vitro folding was found to enhance the folding efficiency when monitored by binding the folded protein to UDP-Agarose (Uridine 5'-Diphospho Hexanolamine) columns. CD and STCD of b4Gal-T5 and b4Gal-T6 were also folded in a step-wise dilution method in the presence of additives PEG/Arg. In the case of STCD, the step-wise dilution method substantially increased the recovery of the folded proteins as compared to the single-step folding method, while it had only a marginal effect on the CD alone. The mutation of the potential glycosylation site, Asn x Ser/Thr in the C-terminal region, to Lys x Ser/Thr, and in addition the deletion of few residues of the C-terminal end, resulted in a substantial increase in the folding efficiency as compared to the respective wild type proteins without any loss of activity. Moreover, the construction of the chimera using the ST region of the b4Gal-T1, fused to the CD of b4Gal-T6, resulted in an increase in the in-vitro folding efficiency compared to CD alone. Enzymatic activity of the folded proteins towards various acceptors showed that b4Gal-T5 and b4Gal-T6 have preferences for the acceptor GlcNAcb-S-pNP (p-nitrophenyl-N-acetyl-1-thio-b-D-glucosaminide) compared to GlcNAc, the preferred substrate for b4Gal-T1. The folded STCD-b4Gal-T5 and STCD-b4Gal-T6 proteins did not show any activity with glucosylceramide as an acceptor substrate. We hypothesize, that b4Gal-T5 and b4Gal-T6 may either require an activator molecule, like α lactalbumin in the case of lactose synthase system, or an additional transmembrane domain for it to show activity with the glucosylceramide acceptor.

(493) **Profiling global O-linked glycosylation in Caenorhabditis elegans using Azidosugar Metabolism**

Scott T Laughlin[1] and Carolyn R. Bertozzi[1,2,3]
[1] Departments of Chemistry, [2] and Molecular and Cell Biology, University of California, Berkeley, [3] Howard Hughes Medical Institute.

The nematode *Caenorhabditis elegans* is an ideal model organism for developmental studies due to its rapid growth, precisely programmed cell lineage, sequenced genome, and transparent physiology. Glycobiologists have recently utilized *C. elegans* to investigate the functions of two major classes of glycans, namely glycosaminoglycans (1) and *N*-linked glycans (2). By contrast, inquiry into the functions of *O*-linked glycans has been stifled by a lack of analytical techniques for enrichment, detection, and cleavage. The ability to monitor global *O*-linked glycosylation will likely initiate the discovery of glycans crucial to an organism's development and survival. Recently, our lab has developed a new technology that enables the probing of mucin-type *O*-linked glycans with an unnatural azide containing *N*-acetylgalactosamine analog (3). Here we report that this azidosugar metabolism can be used for the detection and enrichment of *O*-linked glycans in *C. elegans*. This permits the profiling of differential *O*-linked glycosylation throughout the worm's development and will likely promote progress towards elucidating the role of *O*-linked glycans in eukaryotes.

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(494) **Optimized Characterization of N-linked Glycan Diversity in the *Drosophila* embryo**

Michael Tiemeyer, Kazuhiro Aoki, Shermea Love and Lance Wells
Complex Carbohydrate Research Center, University of Georgia, Athens, GA.

Antisera raised against Horseradish Peroxidase (HRP) cross-react with a family of N-linked oligosaccharides found in a broad range of arthropods, including *Drosophila melanogaster*. Expression of the HRP-epitope in *Drosophila* is tissue-specific, restricted primarily to neural tissue in the embryo and adult. To characterize the diversity of HRP-epitope(s) in embryos, N-linked oligosaccharides were released from preparations of de-lipidated *Drosophila* proteins. The released glycans were fractionated into acidic and neutral populations and screened as neoglycolipids by TLC-overlay. Multiple cross-reacting oligosaccharide species were detected in both the acidic and neutral pools. To further understand the specificity of HRP-epitope expression and to develop methods that will ultimately allow for sensitive detection of glycan expression differences in mutant backgrounds, methods have been optimized to characterize total N-linked glycan diversity in the *Drosophila* embryo. Comparative analysis has been performed to determine optimal Pronase digestion conditions for maximizing glycan release and recovery with PNGaseA or PNGaseF. As expected, PNGaseA release is dependent on peptide length; maximal Pronase digestion does not favor complete glycan release. The N-linked glycan pools released by these enzymes under optimal conditions are overlapping and non-identical, reflecting the inability of PNGaseF to release glycans bearing GlcNAc substituted with Fuc in alpha-3 linkage. Upon 2-AB labeling and HPLC separation (amide column), N-linked profiles resulting from PNGaseA digestion exhibit Fuc-substituted and non-substituted high-mannose oligosaccharides as well as additional, less prevalent species that are amenable to complete characterization upon scale-up. The characterization protocol is currently capable of providing informative profiles on proteins extracted from as little as 500 mg wet weight equivalent of embryos. With only modest scale-up, this amount of starting material can be obtained using existing *Drosophila* embryo collection techniques, providing a means to investigate glycosylation changes in the context of numerous developmental mutations.

(495) **The Behavior of Xylanase in Xylosyl Serine Linkage between a Core Peptide and a Glycosaminoglycan Chain**

Nobuyuki Ibori[1], Mito Iwafune[1], Atsushi Kon[1], Ikuko Kakizaki[1], Atsushi Kuno[2], Satoshi Kaneko[3] and Keiichi Takagaki[1]
[1] Department of Biochemistry, Hirosaki University, School of Medicine, 5

Zaifu-cho, Hirosaki, Aomori 036-8562, Japan, [2] Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan, [3] National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan.

[Purpose] In the development of glycotechnology, the enzyme that can release intact glycosaminoglycans (GAGs) from the proteoglycan, is a useful tool. Xylanase hydrolyzes β-1,4-glycosidic bonds within the xylan backbone, producing β-anomeric xylooligosaccharides. A large amount of both wild type and mutant *Streptomyces olvaceoviridis* E-86 family 10 Xylanase (GH10) proteins can be produced by genetic manipulation. We researched the action of Xylanase in xylosyl serin linkage between a core peptide and a GAG chain.

[Methods] The Endo-β-xylosidase activities of wild type GH10, mutants Cat303 lacking Xylose binding domain with only catalytic domain, Q88A which is mutant of Cat303 mutated easy to recognize Glucose in its subsite -2, and xylanase from *Streptomyces lividans* (GH11) was measured. 4-Methylumbelliferyl-GAG (GAG-MU) was used as a substrate. After the reaction had been terminated, liberated MU from GAG-MU was measured with a fluorescent spectrophotometer. The hydrolysis activities of native peptide-GAGs (peptide-chondroitin sulfate, peptide-dermatan sulfate, and peptide-heparan sulfate) was assessed by HPLC.

[Result] GH10 hydrolyzed xylosyl serin linkage of peptide-GAGs. Hydrolysis activity was greater when short GAG chain was used as a substrate. The enzyme acted on any kind of GAG. The activity of Cat303 was the highest. The activity of GH11 was lower than GH10. The activity of Cat303 was decreased when Xylose binding domain was added in reaction mixture.

[Conclusion] GH10 acts on xylosyl serin linkage between a core peptide and a GAG chain of peptide-GAGs. It is suggested that GH10 mutant will become a useful tool in the development of glycotechnology.

(496) Tandem Mass Spectrometry Approach for Sequencing Dermatan Sulfate Oligosaccharides

May Joy C. Miller, Catherine E. Costello and Joseph Zaia

Mass Spectrometry Resource, Department of Biochemistry, Boston University School of Medicine, 715 Albany Street, R-806, Boston, Massachusetts 02118.

Dermatan/chondroitin sulfate (DS/CS) proteoglycans are expresssed in many mammalian tissues and the DS forms are predominant in skin. DS is particularly interesting because of the biological processes it is implicated in, such as wound repair, infection, cardiovascular disease, tumorigenesis, and fibrosis. DS contains repeating units of iduronic acid (IdoA) and N-acetylgalactosamine (GalNAc), IdoA(1,3)GalNAc(1-4), and may be sulfated at the 4- and 6- positions of GalNAc and 2-position of IdoA. Epimerization at the 5th position of uronic acid ring in chondroitin sulfate B (CSB) differentiates it from chondroitin sulfate A (CSA) and chondroitin sulfate C (CSC).

Linear GAG chains are composed of three parts: the linker (consisting of GlcA-Gal-Gal-Xyl- attached to serine residue of the core protein), the main body, and the non-reducing end. This study concerns sequencing the main body and the non-reducing end of DS chains.

Methods

CSA (GlcA, GalNAc-4-sulfate), CSB (IdoA, GalNAc-4-sulfate), CSC (GlcA, GalNAc-6-sulfate), and all enzymes were obtained from Seikagaku America/Associates of Cape Cod (Falmouth, MA). Unsaturated oligosaccharides were generated by digestion of DS with chondroitinase ACI and/or ABC, while saturated oligosaccharides were generated by digestion with testicular hyaluronidase. Oligosaccharides were partially purified using a Superdex peptide column (3.2 mm x 30 cm, Amersham Biosciences) and 0.1M ammonium acetate, 10% MeOH mobile phase. Negative mode mass spectra were acquired using an Applied Biosystems/MDS-Sciex API QSTAR Pulsar Qo-TOF mass spectrometer fitted with nanospray ion source. CID spectra were obtained at collision energy of -18V where the precursor ion remained abundant.

Results and Discussion

The variable placement of IdoA and sulfation positions on GalNAc residues poses a challenge in sequencing DS polysaccharide chains by MS. To determine the sulfate position and epimerization, we compared the CID ion abundances of the fragment ions formed from DS relative to those produced from standards with known epimerization and GalNAc sulfate positions. Previous studies have indicated that the abundances of glycosidic bond cleavages formed from CID of DS/CS reflect sulfation positions of GalNAc residues. Moreover, ions formed from ring cleavage of the unsaturated GlcA indicate epimerization. The tetramer series of several DS and decorin enabled us to obtain reference for a single uronic acid with its epimerization state.

CID spectra of the unsaturated tetramers isolated after lyase digestion of CSA, CSC, and CSB, respectively, display ions suggesting sulfate positions and epimerization. Ions B_3^{1-} and Y_1^{1-} reflect CSA-like sulfation position, $^{0,2}X_3^{2-}$ and Y_3^{2-} ions display CSB-like epimerization state, and C_3^{2-} and $(M\text{-}SO_3)^{2-}$ ions indicate CSC-like sulfate position.

The non-reducing tetramer is 18 u higher in mass than the main body. The saturated CS standards of known sulfation position were obtained from hyaluronidase digestion. Characteristic ions B_3^{1-} and Y_1^{1-} indicate CSA-like sulfation pattern, C_3^{2-} and $(M\text{-}SO_3)^{2-}$ ions suggest CSC-like content while Y_3^{2-} ions indicate CSB-like epimerization state. Ring cleavage ions, $^{0,2}X_3^{2-}$, were not observed, suggesting that α,β-unsaturation of GlcA is crucial in the formation of $^{0,2}X_3^{2-}$ ions.

The sulfate positions and epimerization of the hexamer and octamer series of DS and decorin are being determined to test the viability of this method.

(497) Glycoconjugate Profiling of *Drosophila melanogaster* by Western-Blot Analysis

Hidenao Toyoda[1,2,3], Naoko Okano[1], Saori Hosoyama[1], Toshihiko Toida[1] and Akiko Kinoshita-Toyoda[1,2]

[1] Department of Bioanalytical Chemistry, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 263-8522, Japan, [2] CREST, JST, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan, [3] PRESTO, JST, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan.

Drosophila melanogaster is well established as a model organism for genetic analysis. With sensitive analytical methods of sugar chain, *Drosophila* is also a very powerful tool for understanding the function of glycoconjugates. For example, a HPLC system with fluorometric post-column detection was successfully applied to flies bearing mutations in glyco-genes [Toyoda, H., Kinoshita-Toyoda, A., Fox, B., and Selleck, S.B. (2000) *J. Biol. Chem.* 275, 21856-21861]. It was found that mutations in *sugarless* compromise the synthesis of both chondroitin sulfate and heparan sulfate. Defects in *sulfateless* did not affect chondroitin sulfate levels or composition but altered the composition of unsaturated disaccharides from heparan sulfate. A mutation in *tout-velu* did not affect chondroitin sulfate synthesis but reduced all forms of heparan sulfate. These findings demonstrate the utility of *Drosophila* as a model organism for studying the function of glycoconjugates *in vivo*. Although the HPLC method makes flies outstanding system for the investigations of glycosaminoglycans/proteoglycans, very little is known about the other glycoconjugates in *Drosophila*. In addition to the chemical analysis of sugar chains, western-blot analysis by use of lectin staining and immunostaining provides attractive tools to further understanding of basic physiological functions of glycoconjugates. In this study, we carried out glycoconjugate profiling of *Drosophila* by 1D or 2D-electrophoresis with western-blot analysis followed lectin staining and immunostaining. *Drosophila* glycoconjugates were well characterized by SBA, ConA and WGA lectins and the method was applied to flies bearing mutations in glyco-genes. Furthermore, proteoglycans were profiled by western-blot analysis with immunostaining. These methods are planning to be employed in the proteo-glycomics study.

(498) Micro-determination of Glycoconjugates in *Drosophila melanogaster* by High-Performance Liquid Chromatography with Fluorometric Detection

Akiko Kinoshita-Toyoda[1,3], Mikiko Ito[1], Atsushi Suzuki[1], Toshihiko Toida[1], Shoko Nishihara[2,3] and Hidenao Toyoda[1,3,4]

[1] Department of Bioanalytical Chemistry, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 263-8522, Japan, [2] Division of Cell Biology, Soka University, 1-236 Tangi-cho, Hachioji, Tokyo 192-8577, Japan, [3] CREST, JST, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan, [4] PRESTO, JST, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan.

Until recently, glycoconjugates were studied principally in vertebrate systems. However, genetic experiments in *Drosophila melanogaster* established that glycoconjugates are required for normal development of this invertebrate model organism. Previously, a highly sensitive method has been reported for the structural analysis of glycosaminoglycans in *Drosophila melanogaster* using a fluorometric HPLC system for unsaturated disaccharides derived from glycosaminoglycans [Toyoda, H., Kinoshita-Toyoda, A., and Selleck, S.B. (2000) *J. Biol. Chem.* 275, 2269-2275]. Disaccharide compositional analysis was performed and chondroitin

sulfate and heparan sulfate-derived disaccharides were determined from *Drosophila*. It is very interesting that the profiles of disaccharides generated by chondroitinase treatment of *Drosophila* material resemble those generated from treatment of human bikunin, a blood plasma protein component of the inter-alpha-trypsin inhibitor family. Heparin lyases treatment of glycosaminoglycans from *Drosophila* released unsaturated disaccharides bearing *N*-, 2-*O*- and 6-*O*-sulfated species, including mono-, di- and tri-sulfated forms like vertebrates. Further examinations revealed that *Drosophila* showed tissue- and stage- specific modifications of glycosaminoglycans. Therefore, *Drosophila* has been recognized as a useful model organism for the structural and functional study on glycosaminoglycans/proteoglycans. The next question for us to consider is to determine whether *Drosophila* could provide a model system for the other glycoconjugates. In this study, we established a sensitive method for compositional analysis of amino and neutral sugars in very small amount of *Drosophila*. Ten flies were applied to pretreatment procedure, then, under one tenth of the materials were loaded onto a HPLC system for monosaccharide determination. We found that the monosaccharide compositions of *Drosophila* glycoconjugates mainly consist of GalN, GlcN, Fuc, Gal, Man and Xyl as vertebrate systems. Furthermore, we are planning to try micro-determination of N-linked oligosaccharides in *Drosophila* for the investigation of functional glycomics using this excellent model organism.

(499) **Analysis of Glycosaminoglycan Oligosaccharides by Ion-Spray Mass Spectrometry**

Kaoru Kojima[1,2], Atsushi Kon[1], Ikuko Kakizaki[1], Yoshiaki Kudo[1] and Keiichi Takagaki[1]

[1] Department of Biochemistry, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan, [2] KUSHIRO Research Laboratory, KAKUHIRO Co.Ltd, 13-1 Otanoshike, Kushiro 084-0917, Japan.

Proteoglycan, a complex glycoconjugate that is composed of core protein and glycosaminoglycan (GAG) chains, are major compornent of extracellular matrix. It is known that GAG has various important physiological and pathological functions including cell migration, morphogenesis, inflammation, and wound healing. GAGs are sulfated polysaccharides made of repeating uronyl-N-acetylhexsosamine disaccharide units. The length and sulfation pattern of GAG are different in each kind of cell, reflecting the diversities of PG functions. Therefore, exact determination of molecular length and extent of sulfation are crucial for the elucidation of GAG functions. Recently, it has been reported that ion-spray mass spectrometry is useful tool for structural analysis of hyaluronan oligosaccharides (4-52 mer). In this study, we have applied ion-spray mass spectrometry for analysis of GAG including hyaluronan and chondroitin 6-sulfate oligosaccharides. GAG oligosaccharides were prepared by enzyme digestion with testicular hyaluronidase, followed by purification utilizing HPLC (Polyamin-II column). Each oligosaccharide with various molecular weights was applied to quadrupole mass spectrometer equipped with an atmospheric-pressure ionization source (API-100 and 300, PE-SCIEX). All mass spectra of each oligosaccharides were infused into ionization probe and operated in the negative ion mode.Å@As a results, the molecular weight of each hyaluronan oligosaccharide, determined by ion-spray mass spectrometry, coincides with their theoretical mass, and up to 142-mer was applicable to this method. Furthermore, chondroitin 6-sulfate oligosaccharide was labeled with 2-aminopyridine to elucidate its structure and subjected to MS/MS analysis. The presence of the suflate residue was confirmed and its binding site was also exactly determined. Therefore, these results strongly indicated that analysis by ion-spray mass spectrometry is useful method for the structural determination of GAG oligosaccharides.

(500) **Synthesis and Biological Evaluation of Glycopolymer as Shiga toxin Neutralizer**

Koji Matsuoka[1], Atsushi Miyagawa[1], Kiyotaka Nishikawa[2], Miho Watanabe[2], Yasuhiro Natori[2], Eiji Kita[3], Tetsuo Koyama[1], Ken Hatano[1] and Daiyo Terunuma[1]

[1] Dept. Functional Materials Sci., Saitama Univ., Saitama 337-8570, Japan, [2] Dept. Clin. Pharmacol., Res. Inst., International Med. Cent. Jpn, Tokyo 162-8655, Japan, [3] Dept. Bacteriol., Nara Med. Univ., Nara 634-8521, Japan.

Oligosaccharide chains in glycoconjugates such as glycoproteins, glycolipids, and proteoglycans play an important role in biological systems. Globotriaosyl ceramide (Gb3: Galα1-4Galα1-4Glcβ1-Cer), one of the major glycolipids, is known as a carbohydrate-based receptor for Shiga toxins (Stxs), which are produced by Shiga toxin producing *Escherichia coli* (STEC) O157:H7. Stxs are belonging to bacterial AB5 toxin families and are classified into Stx1 and Stx2. The toxins have a number of binding sites in the holotoxin, therefore, the toxin displays multivalency. As to enhancement of week binding efficiency between Gb3--Stx by means of sugar cluster effect, we had reported design and synthesis of a new class of glycodendrimers as the toxin neutralizer [1)] and demonstrated those potential activities against both Stxs in vitro as well as vivo [2)]. One of the glycodendrimers having six trisaccharide moieties of Gb3 and appropriate spacer length from the branching point of the dendritic core scaffold showed complete neutralization potency both Stxs, and rescue of mice infected by native *E. coli* O157:H7. These results strongly suggested that multivalent-type globotriaosyl residues and topologically controlled positions of the sugar residues were key factor. In this paper, we describe the synthetic approach for construction of different type of glycocluster as a potential Stx neutralizer and their biological responses against Stxs as well as *E. coli* O157:H7. In brief, glycopolymers having lactosyl or globotriaosyl residue as the carbohydrate receptor for Stxs were prepared via usual radial polymerization protocol from slightly modified carbohydrate monomers, respectively[3)]. Since these glycopolymers showed efficient solubility in water, biological activities of the polymers for a couple of Stxs were evaluated in homogeneous conditions. The results of the glycopolymers--Stxs interaction in vitro showed high binding affinity and strong inhibitory potency of cytotoxic activities of the toxins. In addition, oral administration of the glycopolymers into mice was tested after infection by *E. coli* O157:H7[4)]. These results will also be presented. References 1) K. Matsuoka, et al., Tetrahedron Lett., 40 (1999) 7839-7842. 2) K. Nishikawa, et al., Proc. Natl. Acad. Sci. USA, 99 (2002) 7669-7674. 3) A. Miyagawa, et al., Carbohydr. Polym. (2004) in press. 4) M. Watanabe, et al., J. Infect. Dis., 189 (2004) 360-368.

(501) **Semisynthesis of Homogeneously Glycosylated Human Interleukin-2**

Yu-Ying, Yang, Thomas J., Tolbert and Chi-Huey Wong

The Department of Chemistry and Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

Human Interleukin-2 (IL-2), also known as T-cell growth factor, is a powerful mediator in immune response. Purified human IL-2 derived from healthy donors was found to exist in three major forms. Two of them were glycosylated polypeptide species (16.5 kDa), in which their glycan structures have been identified as NeuAc(£\2-3)Gal(£]1-3)GalNAc and NeuAc(£\2-3)Gal(£]1-3)[NeuAc(£\2-6)]GalNAc. The third major form (14.5kDa) is unglycosylated and has been utilized to treat cancer patients with metastatic melanoma and renal cell carcinoma. Several experimental results have shown that modifying unglycosylated IL-2 with polar groups, sugars for example, can significantly improve its solubility at neutral pH and increase half-life time in plasma. These improved physical properties of human IL-2, which may lead to low-dosage regimen in its clinical use, were expected to be able to ameliorate the dose-related adverse effects happening in those cancer patients administrated with unglycosylated IL-2. Our interest is to attach IL-2 protein fragment with its native sugars, and explore their function in stimulating various immune cells. To this end, various glycopeptide thioesters were synthesized and chemical native ligation was employed to chemoselectively couple these synthetic glycopeptide thioesters to the expressed protein IL-2 fragment. An established quantitive IL-2 assay will be used to examine the bioactivities of these glycosylated IL-2.

(502) **Development of Methodology to Identify Cell-Surface Glycoproteins**

Vinita Marathe and Bruce.A Macher

San Francisco State University,1600 Holloway Avenue, San Francisco, CA-94132.

Cell surface proteins are important therapeutic targets and have been exploited for targeted treatment in several diseases including cancer. Thus, identification of cell surface proteins as therapeutic targets has been a prime area of interest in the proteomics field. However, technical challenges have hampered efforts to effectively isolate and identify cell surface proteins. Numerous studies in the literature have shown that many cell surface proteins are glycosylated. Therefore, a strategy integrating lectin affinity chromatography into a proteomics approach for the identification of cell surface proteins would seem to have merit. We present an initial assessment of protocols that use a selection of lectins to enrich for membrane glycoproteins that can be effectively coupled with protein identification via

ESI-MS/MS. Observations related to cell solubilization approaches, chromatographic conditions and mass spectrometric analysis will be presented. This work was supported by funds from a grant (5P20-MD000262) from the National Center on Minority Health and Health Disparities.

(503) **Structures of Multi-sulfated Oligosaccharides Carrying HNK-1 Carbohydrate Epitopes from Adhesion Protein P0 in peripheral Nerve Myelin.**

Kunio Kitamura, Jianhong Yan, Kayo Fujimaki, Megumi Kumagai and Masahiko Nomura

Dept. Physiol, Saitama Medical School, Iruma-gun, Saitama, 3500495 JAPAN.

Sulfated glucuronic acids is now known as the carbohydrate epitope to the HNK-1 antibody, which was originally shown to react with natural killer cells and identifies an antigen shared by the immune system and the nervous system. In the nervous system, the HNK-1 epitope is expressed on glycolilpids, proteoglycans, and glycoproteins that have been implicated in adhesion or cell-cell recognition in external environment, such as N-CAM (neural cell adhesion molecule) and MAG (myelin associated glycoprotein). This HNK-1 reactive structure has attracted considerable interest both with regard to cell-cell interactions and demyelinating diseases. Even though there are many HNK-1 reactive glycoproteins and proteoglycans, little is known about oligosaccharide structures containing the HNK-1 carbohydrate, except for glycolilpids and P0 and PAS-II/PMP22 glycoproteins from peripheral nerves.

This time, we focused on the acidic oligosaccharides from P0 protein and found extremely acidic glycans, which contain the HNK-1 carbohydrate epitope(s). Among them, we determined the structures of two novel glycans as follows.

MS1: {3SO_4}GlcAβ1-3Galβ1-4GlcNAcβ1-2Manα1-6({3SO_4}GlcAβ1-3Galβ1-4[{6SO_4}GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc.

MS2: Manα1-3Manα1-6({3SO_4}GlcAβ1-3{6SO_4}Galβ1-4{6SO_4}GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc.

In addition to the 3-O-sulfate on the HNK-1 glycans, 6-O-sulfated N-acetylglucosamine residue and 6-*O*-sulfated galactose residue were found in these oligosaccharides. These results suggested that both GlcNAc 6-O-sulfotransferase and 6-*O*-sulfotransferase must be in the peripheral nervous tissue.

(504) **Genome-wide Evolutionary Analyses of Sialic Acid Biology - Evidence for Multiple Differences between Humans and Chimpanzees.**

Tasha Altheide[1], Toshiyuki Hayakawa[1], Tarjei Mikkelsen[2] and Ajit Varki[1]

[1] Glycobiology Research and Training Center and Departments of Medicine and Cellular & Molecular Medicine, University of California at San Diego, La Jolla, CA, USA, [2] The Broad Institute/MIT, Cambridge, MA, USA.

Sialic acids (Sias) are 9-carbon sugars found at the ends of sugar chains attached to cell surface and secreted molecules of vertebrates. In this location, they can mediate recognition by multiple receptors of intrinsic or microbial origin. Of the few known functional genetic differences between humans and great apes, several involve genes affecting Sia biology (e.g., *CMAH, SIGLECL1, SIGLEC9* and *SIGLEC13*). Since all major loci involved in Sia biology have been conserved between human and mouse, availability of the draft chimpanzee genome sequence allows a global analysis of this system during hominoid evolution. High quality chimpanzee orthologs were identified for 46 of the 48 human genes known to be involved in biosynthesis, activation, transport, modification, recycling, degradation, or intrinsic recognition of Sias. Chimpanzee genome sequences were aligned to human and mouse orthologs and modified as needed to maintain an orthologous open reading frame. Overall averages for human-chimpanzee nucleotide and amino acid divergences and Ka/Ks ratios of these genes were similar to the reported genome-wide averages. However, there is evidence for species-specific accelerated evolution at several loci. We confirm and extend prior examples of human-chimp differences, as similar comparisons of several rodent orthologs suggest more rapid evolution of this system between humans and chimps than between mouse and rat. The group of genes involved in Sia recognition appears to be the most rapidly evolving class, as measured by % amino acid divergence and Ka/Ks ratios, consistent with their functional location at the cell surface, and outside the core Sia biosynthetic pathways. This finding includes evidence for accelerated evolution in the Sia binding domains of the Siglec family of intrinsic Sia receptors, which are among the fastest evolving groups of genes in the entire genome. Within a overall vertebrate framework, several other loci also show human- or chimpanzee-specific amino acid differences. In particular, we found specific amino acid changes in otherwise highly conserved functionally important sialylmotifs of some sialyltransferases, including one that is involved in mediating neural plasticity. In addition, human-specific changes were noted in a domain of complement factor H known to be involved in the 'self-recognition' of Sia. Thus, major selective pressures have likely affected Sia biology during hominid evolution and the current human condition may reflect unique adaptive changes in this system.

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(505) **Structural Analyses of ^{3}H-Monosaccharide-Labeled Glycans Derived by Metabolic Radiolabeling of Murine Leukocyte Cell Lines**

Ziad Kawar[1], Yunfang Man[2], John B. Lowe[2], Annette Fleshman[1], Thomas K. Johnson[1] and Richard D. Cummings[1]

[1] Dept. of Biochemistry & Molecular Biology; Oklahoma Center for Medical Glycobiology; and the Consortium of Functional Glycomics Core C and Core H, The Univ. of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, [2] Dept. of Pathology, Life Sciences Institute, and Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI.

As part of the effort by the Consortium of Functional Glycomics to characterize the repertoire of glycan structures present in various cell types, we are using metabolic-radiolabeling of cultured cells with ^{3}H-monosaccharide precursors (Man, GlcNH_2, Fuc) to aid in these analyses. In these approaches, radiolabeled N- and O-glycans are released from total cell tryptic glycopeptides by chemical and enzymatic approaches. Released glycans are separated and structurally analyzed using HPLC approaches, ion-exchange chromatography, and serial lectin-affinity chromatography. Synthetic standard radiolabeled N- and O-glycans were prepared for comparative purposes. These studies allow us to gain a quantitative overview of the structures present in a certain cell type, and to define specific glycan structures that may represent too small a fraction of the total glycans to be clearly identified by mass-spectrometric analyses. In characterizing minor glycan structures, we place particular emphasis on glycoprotein ligands such as PSGL-1. The cells we have analyzed include the murine leukocyte cell line Wehi and immortalized murine leukocyte progenitor cell lines. Among our findings is that the leukocyte progenitor cell line from mice lacking the alpha1,3-fucosyltransferaseVII gene produces N-glyans with a structural pattern essentially identical to glycans from an immortalized murine leukocyte control line, except for differences in alpha2,6-sialylation. Here we will present some of our most recent data from several cell-lines and specific glycoproteins.

(506) **Targeting RNAs by Tobramycin Analogs**

Fu-Sen Liang, Sheng-Kai Wang , Takuji Nakatani and Chi-Huey Wong

Department of Chemistry and Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA.

Aminoglycosides, a family of structurally diverse aminosugar and aminocyclitol conjugates, are known as potent antibiotics targeting bacterial ribosomal RNA 16S A-site, and are also been found to bind several different viral and human RNA sequences. Tobramycin is a 4, 6-linked

aminoglycoside containing the commonly found two-ring core structure. Tobramycin analogs were designed by replacing the third aminosugar ring with various mono- and di-saccharide in order to find potent and specific RNA binders. A variety of thioglycoside building blocks were glycosylated to the 6-position of the nebramine core, which was derived from tobramycin, to afford tri- or tetra-saccharide analogs. Deprotected tobramycin analogs were tested against several disease-related bacterial, viral and human RNAs by surface plasmon resonance. Several analogs were found to have higher affinity and specificity.

(507) **Synthesis of a Bacterial Glycolipid as a Natural Antigen for CD1d-Restricted T Cells**

Douglass Wu[1], Guo-wen Xing[1], Yuki Kinjo[2], Mitchell Kronenberg[2] and Chi-Huey Wong[1]

[1] Department of Chemistry and the Skaggs Institute of Chemical Biology, The Scripps Resarch Institute, 10550 N. Torrey Pines Rd. La Jolla CA 92037, [2] Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, San Diego CA 92121.

The CD1 family of proteins bind self and foreign glycolipids for presentation to CD1-restricted T cell receptors. A bacterial glycolipid, α-galacturonosyl-ceramide, and three analogues have been synthesized. They show to have immunological activity similar to the well-known CD1d ligand, α-galactosyl-ceramide (a-GalCer) as measured by IL-2 secretion. Structurally, it most significantly differs with a-GalCer at the 6' OH of the sugar moiety along with minor differences in the acyl tails. This bacterial glycolipid is believed to be involved in CD1d mediated T cell activation.

(508) **Semantic Integration and Analysis of Glycomics Information**

William S. York[1], Amit Sheth[2], Krystof Kochut[2], John A. Miller[2], Christopher Thomas[2], Karthik Gomadam[2], Xiaochuan Yi[2], Meenakshi Nagrajan[2] and Satya S. Sahoo[2]

[1] Complex Carbohydrate Research Center, University of Gerogia, Athens GA 30602 USA, [2] Large Scale Distributed Information Systems Laboratory, University of Georgia, Athens GA 30602, USA.

It is often possible to interpret an individual experiment involving a complex biological system only if the data obtained is evaluated in a broad context that takes into account diverse information obtained by several techniques. A large amount of interpreted experimental data is becoming available over the internet, making it possible to collect diverse information and permitting broadly based hypotheses to be generated and evaluated. However, selection of the relevent pieces of information will be difficult, due to the sheer volume of data that is available. The integrated semantic methodology that we are developing to address this challenge utilizes ontology driven procedures, with glycomics as the application domain. A set of interdependent ontologies called GlycO (i.e., a Glycomics Ontgology) is being generated to contain extensive domain knowledge that includes semantically rich descriptions of glycan structure, glycan binding relationships, glycan biosynthesis, and the developmental biology of stem cells. Methods are being developed to utilize multiple, heterogeneous (structured and semi-structured) sources for the automated population of these ontolgies with knowledge, including, for example, specific glycan structures and the building blocks (carbohydrate residues) from which these structures are assembled. The sources of this information and an indication of the reliability of the information (i.e., provenance) is also an integral part of the system. Tools for visualizing and browsing the ontolgies, forming and executing relevant semantic queries, and annotating text documents and databases by reference to ontological classes are being developed. Together, these semantic structures, metadata, and tools comprise the foundation for the evolution from a database model, in which information is simply retrieved for human analysis, to a knowledgebase model, in which interactive and automated knowledge discovery is used for exploring and interpreting complex biological data.

(509) **Evolution of Lactose and Milk Oligosaccharides; A Hypothesis**

Tadasu Urashima[1] and Michael Messer[2]

[1] Department of Life Science & Agriculture, Obihiro University of Agriculture & Veterinary Medicine, Inada cho, Obihiro, Hokkaido, 080-8555, Japan, [2] Department of Biochemistry, The University of Sydney, NSW, 2006, Australia.

Mammalian milk or colostrum contains a few percent carbohydrates, of which disaccharide lactose usually makes up more than 80%. Free lactose is synthesized within lactating mammary glands from UDP-galactose (donor) and glucose (acceptor) by a transgalactosylation catalyzed by a complex of a É¿4galactosyltransferase Öü (É¿4GalTÖü) and Éø-lactalbumin (Éø-LA), one of the milk proteins. As other tissues do not contain Éø-LA but do contain É¿4GalTÖü, the expression of Éø-LA within the mammary gland is the key to the presence of lactose in milk. Éø-LA is believed to be evolved from c-type lysozyme. Mammalian milk or colostrum usually contains less than 20% of milk oligosaccharides, which consist of many saccharides with a lactose unit in the reducing end, too, as well as lactose, while the milk of monotremes, marsupials, and bears, a eutherian species, contains more milk oligosaccharides than lactose. The following hypothesis is proposed for the evolution of lactose and milk oligosaccharides. The proto-lacteal secretions of the primitive mammary glands of the common ancestor of mammals contained fat and protein but not lactose and milk oligosaccharides because of non-expression of Éø-LA. When Éø-LA first appeared, its content within the lactating mammary glands was low and lactose was synthesized at a relatively slow rate. Because of the presence of glycosyltransferases, almost all of the lactose was utilized for the synthesis of oligosaccharides. The predominant saccharides in the proto-lacteal secretions or milk produced by this common ancestor were oligosaccharides and not free lactose. Initially, the oligosaccharides served mainly as anti-infection factors against pathogenic microorganisms alomg with other defense factors. They were then recruited as an energy source for the neonates; this was achieved by an increase in the synthesis of Éø-LA and possibly also of the glycosyltransferases. The two biological roles of anti-infection and provision of energy were preserved in both monotremes and marsupials but in most eutherians the milk concentration of free lactose increased due to a significant increase in Éø-LA synthesis by the mammary gland. Lactose therefore became a significant energy source for most eutherians while oligosaccharides continued to serve mainly as anti-microbial agents. The advent of milk lactose at relative high concentrations necessitated the prior evolution or co-evolution of brush border small intestinal lactase, which provided an efficient mechanism for the digestion of lactose.

(510) **Galactosyllactoses in the Milk of Japanese Women: Changes in Concentration during the Course of Lactation**

Wataru Sumiyoshi[1], Tadasu Urashima[2], Tadashi Nakamura[2], Ikichi Arai[2], Takashi Nagasawa[3], Tadao Saito[4], Norihiko Tsumura[5], Bing Wang[6], Janette Brand-Miller[6], Yoko Watanabe[6] and Kazumasa Kimura[6]

[1] Life Science Research Center, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa, 761-0793, Japan, [2] Department of Bioresearch Science, Obihiro University of Agriclture & Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, 080-8555, Japan, [3] Department of Agro-bioscience, Faculty of Agriculture, Iwate University, Ueda 3-chome, Morioka, Iwate, 020-8550, Japan, [4] Department of Bioproduction, Graduate School of Agriculture, Tohoku University, Tsutsumidori-Amamiya machi 1-1, Aoba-ku, Sendai, Miyagi, 981-8555, Japan, [5] Department of Obstetrics and Gynecology, Asahikawa City Hospital, Kinsei-cho, Asahikawa, Hokkaido, 070-8610, Japan, [6] Human Nutrition Unit, Department of Bichemistry, University of Sydney, NSW, 2006, Australia, [7] YAKULT Central Institute for Microbiological Research, 1796 Yaho, Kunitachi, Tokyo, 186-8650.

The concentrations of three galactosyllactoses found in the milk of 24 Japanese women were determined using normal phase high performance liquid chromatography of the 2-aminopyridyl (PA) derivatives of the oligosaccharides. The milk was collected at 4, 10, 30, 100 days postpartum. The concentration of 6'-galactosyllactose (6'-GL) was maximal at 4 days postpartum, and then decreased, whereas the concentrations of 3'- and 4'-galactosyllactose (3'-GL and 4'-GL) did not change during the course of lactation. In human milk, 6'-GL was the main oligosaccharide among these galactosyllactoses at 4 days postpartum, in contrast to bovine milk in which 3'-GL has been reported to be predominant. The concentrations of these galactosyllactoses in human milk were much lower than those of reported for 2'-fucosyllactose, 3-fucosyllactose, lacto-N-fucopentaose áT, áU, áV, and 3'- and 6'-N-acetylneuraminyllactose.

(511) **Oligomannose-Coated Liposomes Induce T-Helper Type 1 Responses against Various Encased Antigens, which can Control Diseases Progression, in BALB/c Mice**

Yoshitaka Shimizu[1,2], Toru Yoshii[3], Le Biao[1] and Naoya Kojima[1,2,3]

[1] Institute of Glycotechnology, Tokai University, [2] CREST, JST, [3] Department of Applied Biochemistry, Tokai University, Japan.

Cell-mediated immunity is implicated to be involved in protection against infections of virus and parasites such as *Leishmania major*. Resistance or susceptibility against infections depends on the expansion of one of two distinct subsets of CD4+ lymphocytes, T helper-type 1 (Th1) cells or Th2 cells. In the case of several infectious diseases including *L. major* infection,

Th1 CD4+ cells are involved in prevention of infections. Therefore, if a Th1 response can be controlled, these kinds of infections can be prevented. We synthesized a neoglycolipid consisting of oligomannopentaose and dipalmitoylphosphatidylethanolamine and recently demonstrated that soluble leishmanial antigen (SLA)-encapsulated liposomes coated with the neoglycolipid induce protective response to *L. major* infection in BALB/c mice. However, the critical mechanisms of the protective effect of oligomannose-coated liposomes have yet to be clarified. This study investigated the immunoresponse as determined by cytokine profile in BALB/c mice that received the oligomannose-coated liposomes in immunized mice. The mice were intraperitoneally or subcutaneously immunized with oligomannose-coated liposome encased with either SLA, SIV-gag, or crude extracts of EL4 cells. In response to *in vitro* stimulation with each encased antigen, spleen cells from mice that received these antigen-encased oligomannose-coated liposomes had greater production of Th1 cytokines such as IFN-É¡, IL-12, and IL-2 than did those from mice receiving the antigen alone or antigen-encased non-coated liposomes. These results indicate that the antigen-specific Th1 response was predominantly induced in the mice receiving oligomannose-coated liposomes. During the course of subsequent infection with *L. major*, the BALB/c mice receiving oligomannose-coated liposomes showed effective control of the disease as assessed by mild footpad swelling and antigen-specific Th1 response as assessed by dominant production of IFN-É¡. Intraperitoneal injection of bone marrow-derived macrophages, which had been pulsed with oligomannose-coated SLA-encased liposome *in vitro*, led to reduction of footpad swelling of the mice. Since it has been shown that intrapeitoneal macrophages uptake the oligomannose-coated liposomes specifically and effectively (see accompanied paper, Ikehara et al.), uptake of the antigen-encased oligomannose-liposome into antigen presenting cells may trigger induction of the antigen-specific Th1 response in BALB/c mice. Our results suggest that the oligomannose-coated liposomes led to induction of a long-term Th1 response specific for the encapsulated antigen and that these liposomes could be useful as adjuvants against infectious diseases that require cellular immunity to prevent infection. This work was supported in part by a grant for Hi-Tech Research from Tokai University and a grant for AIDS research from the Ministry of Health, Labor, and Welfare of Japan (NK).

(512) N-Glycans on GM3 Synthase Require for Activity But Can Be Substituted with Specific Amino Acids at or near the Glycosylation Sites

Satoshi Uemura[1], Tomoko Suzuki[1], Makoto Ito[2], Masaki Saito[3], Yasuyuki Igarashi[1] and Jin-ichi Inokuchi[1]

[1] Grad. Sch. of Pharm. Sci., Hokkaido Univ., [2] Grad. Sch. Biores. Bioenviron. Sci., Kyushu Univ., [3] Pharmacodynamics, Meiji Pharm. Univ..

The GM3 syntahse (SAT-I) is one of the sialyltranferase, which transfer a sialic acid to lactosylceramide. In this study, we have investigated the role of N-glycans on SAT-I by measuring the in vitro enzyme activity after transfection of SAT-I cDNA and its mutants into CHO cells. There are some N-glycosylation sites in SAT-I, but they are not conserved among the species, such as human, mouse, rat, and zebra fish, expect for the site in their sialylmotif L. By using a series of inhibitors for N-glycans processing, we have clarified that N-glycans of high mannose type are essential for SAT-I activity, since treatment of tunicamycin resulted in the complete loss of the activity but those of kifnecine and castanospermine had not effect. By examing the mutants replaced asparagines with glutamine (N180Q, N224Q, N334Q in mouse SAT-I (mSAT-I)), we identified that all of three sites are N-glycosylated, and each N-glycans is required for the activity. Next we asked whether the function of N-glycans on SAT-I activity could be substituted with specific amino acid residues, which have varied among species, at or near the glycosylation sites. To this end, we constructed a series of inter-species mutants of mSAT-I, N224K (human type) and T336Q (zebra fish type). In the case of N-glycosylation sites in sialylmotif L, which is preserved among the species, we constructed mSAT-I-H177D-N180S mutants, which is equal to the sequence of related sialyltransferases, STGal III and STGal IV. The SAT-I activity of these mutants was quite similar to that of wild-type. Thus, we could demonstrate here that N-glycans on SAT-I requires the enzyme activity but can be substituted with specific amino acid residues at or near the glycosylation sites.

(513) A New Bioorthogonal Ligation for Labelling of Azide-Bearing Glycoconjugates

Nicholas J. Agard, Jennifer A. Prescher and Carolyn R. Bertozzi

Departments of Chemistry and Molecular and Cellular Biologyt, University of California, Berkeley, Berkeley, CA 94720

Selective chemical reactions that are orthogonal to the diverse functionality of biological systems have become fundamental tools for the chemical biologist. As relative newcomers to the repertoire of synthetic chemistry, these bioorthgonal reactions have inspired new strategies for protein engineering (1-3), functional proteomics (7), and chemical remodeling of cell surfaces (1,4,5). The azide has secured a prominent role as a unique chemical handle for bioconjugation. We have made extensive use of the azide as a chemical reporter of glycosylation, employing the Staudinger ligation with phosphines to tag azidosugars metabolically introduced into cellular glycoconjugates (6,7). Remarkably, the Staudinger ligation can be performed in living animals without physiological harm (8), suggesting the potential for applications in non-invasive imaging and therapeutic targeting. Still, the reaction is not without liabilities. The requisite phosphines are susceptible to air oxidation and their optimization for water solubility and increased reaction rate has proven to be synthetically challenging. Here we present a novel bioothogonal reaction that can be used to label unnatural azide-bearing glycoconjugates, and that eliminates some of the problems inherent to phosphine based molecules. This ligation utilizes derivatized cyclooctynes which have been shown to react rapidly with azides in [3+2] cylcoadditions. In contrast to linear alkynes, the triple bond of cyclooctyne is bent due to its placement in a medium sized ring. The resulting ring strain destabilizes the ground state and drives the reaction to proceed at a reasonable rate. Cyclooctynes conjugated to biological probes were used to covalently modify azide-labeled glycoproteins and cells expressing unnatural azido-sugars. Additionally we have investigated existing methods to label azide-bearing glycans, and compared them with our new methodology. We are currently using this technology for non-invasive imaging and to proteomic applications. (1) Link, A. and Tirrell, D. A. *J. Am. Chem. Soc.* 2003, 125, 1164-1165. (2) Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.* 2003, 125, 3192-3. (3) Kiick, K. L.; Saxon, E.; Tirrell, D. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U. S. A.* 2002, 99, 19-24. (4) Mahal, L. K.; Yarema, K. J.; Bertozzi, C. R. *Science* 1997, 276, 1125-8. (5) Saxon, E.; Bertozzi, C. R. *Science* 2000, 287, 2007-10. (6) Dube, D. H.; Bertozzi, C. R. *Curr. Opin. Chem. Biol.* 2003, 7, 616-25. (7) Vocadlo, D. J.; Hang, H. C.; Kim, E. J.; Hanover, J. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U. S. A.* 2003, 100, 9116-21. (8) Prescher, J. A.; Dube, D. H.; Bertozzi, C. R. *Nature* (in press).

(514) Gb2 Disaccharide-Coated Sensor Chip for Verotoxin Detection

Masayuki Izumi[1], Hirotaka Uzawa[1], Hiroki Itoh[1], Shin-ichiro Yokoyama[2], Hiroshi Mori[2] and Norihiko Minoura[1]

[1] Research Center of Advanced Bionics, National Institute of Advanced Industrial Science and Technology, 1-1-1 Higashi, Tsukuba, Ibaraki, 305-8565 Japan, [2] Department of Public Health Pharmacy, Gifu Pharmaceutical University, Gifu, Gifu 502-8585 Japan

Escherichia coli O157:H7 is an emerging cause of foodborne illness. *E. coli* O157:H7 infection often causes severe bloody diarrhea and abdominal cramps. These symptoms are the results of the inhibition of protein biosynthesis in ribosome by verotoxins produced by the bacterium. In some persons, particularly children under 5 years of age and the elderly, the infection can also cause a complication called hemolytic uremic syndrome (HUS), in which the red blood cells are destroyed and the kidneys fail. About 2%-7% of infections lead to this complication. HUS is a life-threatening condition usually treated in an intensive care unit. Rapid diagnosis for *E. coli* O157:H7 infection is important for the prevention of this complication and fast recovery. Usually, infection with *E. coli* O157:H7 is diagnosed by detecting the bacterium by cell culture. If detected, the production of verotoxins by the bacterium is tested. These procedures take several days. Here we report the rapid detection of verotoxins using Gb2-disaccharide coated sensor chip.

Verotoxins (shiga toxins; Stxs) are known as a family of AB_5 bacterial toxins and two types of Stxs (Stx-1 and Stx-2) are produced by *E. coli* O157:H7. Multivalent binding of five B-subunits to cell surface Gb3 (globotriaosylceramide) is the first step of Stx-1 infection. Inspired by the specific binding of Stxs and Gb3, we have developed a sensing film consists of a monolayer of monoalkyl globobioside (Gb2). Gb2 is the minimun structure required for the binding of Stxs. The film was produced using Langmuir-Blodgett (L-B) technique, and Stxs were detected by a quartz crystal microbalance (QCM) method (1). However, the film was not stable and needed to be prepared just before use. To develop the stable Gb2-coated sensor chip for Stxs detection, we examined three immobilization methods using chemically synthesized 10-aminodecyl glycoside of Gb2, or Gb2-

containing polyanion which was synthesized by the reaction of 10-aminodecyl Gb2 with poly(ethylene-*alt*-maleic anhydride). The first method was the direct attachment of 10-aminodecyl Gb2 with a chip coated with carboxymethylated dextran. The second method was the covalent attachment of the Gb2-containing polyanion with a chip having amino groups at the surface through amide bond. The third method was the absorption of the Gb2-containing polyanion to a sensor chip through polyelectrolyte multilayer. Using these sensor chips, detection of Stx-1 was examined by a surface plasmon resonance (SPR) method. Sensor chips produced by the first two methods were not sensitive enough (detection limit: 5 μg/mL) probably because of the low density of Gb2. Using the sensor chip produced by the third method, Stx-1 could be detected at as low concentration as 100 ng/mL, which is lower than the LD_{50} value of Stxs in blood (250 ng/mL). It is noteworthy that the response of this sensor chip did not change after 24 h.

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(515) **Single Step Multi-syntheses of Glycosyl Acceptors : Benzylation of *n-1* Hydroxyl Groups of Phenylthio-glycosides of Xylose, Mannose, Glucose, Galactose, 2-Azido-2-deoxyglucose, 2-Azido-2-deoxygalactose**

Kaori Suzuki, Isao Ohtsuka, Takuya Kanemitu, Takuro Ako and Osamu Kanie

Mitsubishi Kagaku Institute of Life Sciences (MITILS), 11 Minamiooya, Machida-shi, Tokyo 194-8511 Japan

The cell surface is covered with a variety of oligosaccharides which are anchored to lipids and proteins. Oligosaccharides play important roles in cellular recognition process. Such processes include differentiation and development of cell, immune response and fertilization in the animal kingdom. Other functions are the role as ligands of receptors present on the surfaces of bacteria and viruses causing infection diseases. In order to access functions and mechanism involved in such interactions, synthetic oligosaccharides can be used as molecular probes. Also it is important to synthesize an oligosaccharide library. In order to access such molecules, one has to synthesize variety of monosaccharide synthetic units required to construct oligosaccharides. In most cases, it is relatively easy to synthesize such units, but they usually requires three to five steps for just protecting group manipulations and thus time consuming in other words expensive. In the course of our study to synthesize oligosaccharide library, it was necessary to prepare a series of suitably protected monosaccharide synthetic units. To rapidly access these protected monosaccharides, we considered to introduce *n-1* protecting groups into monosaccharides having *n* hydroxyl groups in a random fashion to yield a set of monosaccharide acceptors. Here we report a result of benzylation of phenylthio-glycoside of monosaccharides - such as xylose (Xyl), mannose (Man), glucose (Glc), galactose (Gal), 2-azido-2-deoxyglucose (GlcN), 2-azido-2-deoxygalactose (GalN) under phase transfer condition to directly prepare glycosyl acceptors. Unlike usual methods, our objective is to obtain multiple products in shortest steps necessary. For this reason, we have tried to 'optimize' the conditions for requirements -1) *n-1* hydroxyl groups are benzylated and 2) expected abundance of individual product is equal. The following represents general methods.

1.25~1.5 equivalents of benzyl bromide (BnBr) for each hydroxyl group, tetra-n-butyl ammonium hydrogensulfate (Bu_4NHSO_4) as catalyst and sodium hydroxide were used in a dichloromethane-water solvent system under gentle reflux conditions at bath temperature of 50 degree. It was shown that the desired compounds, which have one hydroxyl group and other hydroxyl groups were benzylated were produced in relatively scattered ratio.

As a conclusion random benzylation method of phenylthio-glycosides of monosaccharide was examined to rapidly access a set of glycosyl acceptors. It was found that phenylthio-GalN, Xyl and Man can be converted into di- and tribenzyl forms in single phase transfer reactions, and also individual compounds in each series could easily be isolated by silica gel column chromatography. HPLC can be used when purification is not possible such as phenylthio-Glc and Gal. Despite some drawbacks in purification, the method dramatically reduce the synthetic steps thus overall yields for most of individual compounds are superior compared to traditional one-by-one and step-by-step methods.

This research was supported by Key Technology Research Promotion Program, The New Energy and Industrial Development Organization (NEDO), Ministry of Economy, Trade and Industry (METI) of Japan.

(516) **Toward Trisaccharide Library**

Takuro Ako, Isao Ohtsuka, Shusaku Daikoku and Osamu Kanie

Mitsubishi Kagaku Institute of Life Sciences (MITILS), Minamiooya 11, Machida-shi, Tokyo 194-8511 Japan.

With the advance of research of the glycolipids and glycoproteins, importance of oligosaccharides is increasingly recognized. Some of the functions of oligosaccharide associates with various biological phenomena such as cell differentiation, generation, intercellular adhesion, and malignant alteration. Structures of oligosaccharide were extremely complicated due to a number of free hydroxyl groups per monosaccharide unit, existence of branching, and configurations (anomeric isomers) as compared with those of peptide and nucleic acid.

With variety of information regarding biological functions of oligosaccharide, it might be possible to find a candidate that is useful as a seed of medicine developments in future. Despite the difficulties in the synthesis, oligosaccharide library has potential in such purpose. In the field of synthesis of peptide and nucleic acid, solid-phase synthesis is well recognized for its rapid accessibility to a desired compound. Synthesis of oligosaccharide library, however, has been substantially slow because structures of oligosaccharide are more complex. In order to address the issue and to open a door to the oligosaccharide library, we decided to synthesize a linear trisaccharide library, which composed of nine monosaccharides existed in our body. We report herein a preliminary investigation of a solid-phase synthesis of a trisaccharide (Fuc-Gal-Glc) where an extremely efficient method for the oligosaccharide solid-phase synthesis based on diffusive reaction is described.

We have carefully chosen an amino TentaGel™ as the resin. The reasons of this decision are followings: the polyethylene / polystyrene based resin swells well in various solvents and resists against various acid / base reaction conditions. Required monosaccharide units were prepared prior to the synthesis. These units were synthesized as phenylthio-glycoside and glycosyl fluoride to suite for the orthogonal glycosylation strategy. All hydroxyl groups, except for one, are protected with benzyl groups, which are stable under various glycosylation conditions.

To begin with solid-phase synthesis, a succinoyl ester of phenylthio-glycoside of fucose unit was introduced onto the resin, yielded a resin-bound fucosyl donor. The following glycosylation reactions of thioglycoside donor and glycosyl fluoride were performed under low temperature (-30~0°C) and diffusive reaction conditions. Activation of phenylthio-glycoside and glycosyl fluoride was achieved using DMTST and $Cp_2Hf(OTf)_2$, respectively. 1-Octanol as a hydrophobic tag was finally incorporated to the trisaccharide, and the product was cleavaged from the resin (NaOMe) and the benzyl groups were removed.

As a result, Fucα/β(1→6)Galα/β(1→6)Glcα/βOctyl and Fucα/β(1→6)Galα/β(1→4)Glcα/βOctyl were synthesized in overall yields of 33% and 18% in 5 steps, respectively. Obtained mixture of 2^3 stereoisomers were separeted by reversed-phase LC/MS, and thus synthesis of a series of linear trisaccharides with all combination of anomers was accomplished.

This research was supported by Key Techology Research Promotion Program. The New Energy and Industrial Development Organization (NEDO), Ministry of Economy, Trade and Industry (METI) of Japan.

(517) **The Design and Application of a Non-Malodorous Thioglycosylation Method**

Kenta Watanabe, Hirofumi Dohi, Yoshihiro Nishida and Kazukiyo Kobayashi

Department of Molecular Design and Engineering, Graduate School of Engineering. Nagoya University, Furocho, Nagoya 464-8604, JAPAN..

Key words

stereoselective glycosylations, thioglycosides, globosyl oligosaccharides, carbohydrates.

Introduction

Thioglycosyl donors have been used in a large number of oligosaccharide syntheses, because of the chemical stability for storage and high chemo-selectivity under glycosylation conditions. They have many merits, but we have hesitated to use the thioglycosylation method, because it evolves unpleasant odor in the process of preparing the thioglycosyl donors and isolating the glycosyl products. The malodorous mercaptanes shoud be unbearable even if the reaction achieves an excellent outcome. We reported non-malodorous thioglycosyl donors with a methyl thiosalycylate leaving group[1), 2), 3)]. It evolved little unpleasant odor during its preparation and

treatment and could be kept as an active glycosyl donor for months without decomposition. It had unique tendency of α-/β-selectivity. Glycosylation with primary alcohol gave β-selective products, but glycosylation with secondary alcohol gave α-selective products. We assumed that *o*-alkoxycarbonyl group plays key roles in the non-malodorous odor and α-/β-selectivity. In this study, we have designed a new class of non-malodorous thioglycosyl donors to enhance reactivity and selectivity. We examined the effect of substituents (ethyl, *n*-propyl, *iso*-propyl and *tert*-butyl ester) in the *o*-carboxylate group and discussed on the reactivity from bulkiness and electron donating effects.

Results & Discussion

We synthesized novel thioglycosyl donors with a thiosalycylate leaving group. o-Methoxycarbonylphenyl 1-thio-β-$_D$-galactopyranoside was synthesized via the S_N2 reaction of penta-*O*-acetyl-α-$_D$-galactopyranosyl bromide with methyl thiosalicylate. Methyl thiosalycylate smells faintly like a blend of methyl thiosalycilate and thiophenol. After demethylation, a new class of thipglycosyl donors was synthesized via respective ways in high yields. Reactivity and stereoselectivity of new thioglycosyl donors were examined using primary and secondary alcohols as an acceptor and NIS/trifluorosulfonic acid (TfOH) as an activator in solvents (CH_2Cl_2, Et_2O/CH_2Cl_2). Glycosylation with primary cetyl alcohol gave β-selective products, but glycosylation with secondary alcohol (glucose derivatives) gave α-selective products. The thioglycoside with *tert*-butyl ester showed higher reactivity and α-selectivity than that with methyl ester. These donors are useful because they are stable for storage and could be synthesized in high yields. The novel thioglycoside with *tert*-butyl ester was applied to the assembly of globosyl oligosaccharides (Gb_2 and Gb_3)derivatives. We have succeeded in the synthesis of Gb_2 and Gb_3 derivatives more efficiently by using the noble thioglycoside with *tert*-butyl ester.

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(518) **Structures of Major *N*-Linked Oligosaccharides Possibly Associated with Bovine Histone H1**

Noriko Takahashi[1,2], Misaki Kanai[3], Kay-Hooi Khoo[4], Koichi Kato[1,2] and Kiyoshi Furukawa[3]

[1] Graduate School of Pharmaceutical Sciences, Nagoya City University, [2] CREST, GST, [3] Department of Biosignal Research, Tokyo Metropolitan Inst. of Gerontology , [4] Inst. of Biological Chemistry, Academia Sinica.

It is not obvious whether or not mammalian histone chromosomal proteins are *N*-glycosylated. When we analyzed a partial amino acid sequence of a 30 K protein derived from human leukemic cell lysate, which reacted with several lectins including Con A, the sequence was included in human histone H1, suggesting that histone proteins are *N*-glycosylated (S. Guo, H. Mori, T. Matsuda, K. Furukawa, unpublished data). Lectin blot analysis of bovine histone H1 preparations from several commercial sourses showed they are positive to Con A. When a commercially available bovine histone H1 preparation was subjected to Con A-Sepharose column chromatography, only a few percent of it bound and eluted from the column with 100 mM α-methyl mannoside. The pass-through and bound fractions gave a single protein band with a molecular weight of 30 K as resolved by SDS-polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining. *N*-linked oligosaccharides were released from the possibly glycosylated histone H1 by digestion with glycoamidase A from almond, and then labeled with 2-aminopyridine. The structures of major pyridylaminated (PA) *N*-linked oligosaccharides were determined by the 3-D HPLC mapping method as described previously [Takahashi et al. (1995) Anal. Biochem. 226, 139-146]. Upon DEAE-Sepharose column chromatography, PA-oligosaccharides were separated into neutral and acidic ones at almost the same ratio. The neutral and acidic oligosaccharides were separately subjected to octadecyl-silica (ODS) column chromatography and then to amide-silica column chromatography. The results showed that both neutral and acidic oligosaccharides contain a series of high mannose-type ones with 5, 6, 7, 8, and 9 mannose residues. When acidic PA-oligosaccharides were digested with phospho-monoesterase, most of them were converted to neutral ones, suggesting that the acidic nature is due to phospate groups and that high mannose-type oligosaccharides are phosphorylated. Mass spectrometric analysis of the two major acidic oligosaccharide peaks obtained by 2-D HPLC method showed that they include the $Hex_6HexNAc_2$ and $Hex_5HexNAc_2$ structures, respectively. These results suggest that high mannose-type and phosphorylated oligomannosidic sugar chains are associated with bovine histone H1. Lectin blot analysis of other bovine histone subfractions, which are also commercially available, showed that a very small portion of each histone subfraction (H2A, H2B, H3 and H4) appears to be glycosylated. Since histone proteins are considered not to have signal sequences required for entry to the ER-Golgi pathway, how and where they are glycosylated in cells remains to be determined before reach the solid conclusion together with the confirmation of the above phenomenon in other animal species.

(519) **Synthesis Based on Affinity Separation Using Interaction between Podand Type Tag and Ammonium Ion: Application to Oligosaccharide Synthesis**

Mamoru Takashina, Yumiko Hori, Shoichi Kusumoto and Koichi Fukase

Department of Chemistry, Graduate School of Science, Osaka University, Machikaneyama 1-1, Toyonaka, Osaka 560-0043, Japan.

We previously reported a new hybrid methodology of solid-phase synthesis and traditional solution synthesis, which was termed "synthesis based on affinity separation (SAS)". In this methodology, the reaction is carried out in solution and a desired tagged compound is separated from a reaction mixture by solid-phase extraction using specific molecular recognition of a tag. We initially employed the interaction between a crown ether tag and polymer-supported ammonium ion for SAS. Aminomethylated polystyrene resin ArgoPore™ [trifluoroacetic acid (TFA) form] was used as a column stationary phase. In the present study, we elaborated a new SAS method using podand-type tag, a pseudo-1,3-benzo-31-crown-10 structure, and applied it to oligosaccharide synthesis. The podand tag was much easier to synthesize than the corresponding crown ether. The tagged compound is retained in the column by using nonpolar solvents such as dichloromethane and toluene, whereas other excess reagents and by-products are washed out of the column. The desired tagged compound is then eluted from the column by using polar solvents such as methanol-dichloromethane as an eluent. The podand moiety was attached to glucose and galactose residue via an acylaminobenzyl or an acylaminophenyl group, respectively. After glycosylation of tagged glucose with glycosaminyl trichloroacetoimidate, the reaction mixture was subjected to the affinity separation. The desired compounds possessing the podand tag were effectively separated by the affinity between podand and ammonium ion. The resulting disaccharide was reacted with glycosaminyl or galactosyl trichloroacetoimidate to give corresponding trisaccharides.Å@The acylaminobenzyl linker was then effectively cleaved by DDQ oxidation. On the other hand, the acylaminophenyl linker was cleaved by CAN oxidation. In summary, we have developed a new synthetic strategy based on affinity separation. The present method proved to be useful in oligosaccharie synthesis. The strategy greatly facilitates the purification procedures in solution-phase synthesis and is expected to be particularly useful for multiple parallel synthesis and combinatorial library preparation where application of solid-phase synthesis is difficult.

(520) **Host Recognition of Peptidoglycan by Intracellular Receptor Nod1 and Nod2, Investigation with Synthetic Partial Structures**

Akiko Kawasaki[1], Seiichi Inamura[1], Atsushi Shimoyama[1], Naohiro Inohara[2], Gabriel Nunez[2], Yukari Fujimoto[1], Koichi Fukase[1] and Shoichi Kusumoto[1]

[1] Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan, [2] Department of Pathology, The University of Michigan Medicinal School, Ann Arbor, Michigan 48109, USA.

Bacterial cell wall peptidoglycan (PGN) has been well-known as a potent immunopotentiater and adjuvants for antibody production. PGN consists of polysaccharide chains linked to a peptide network to form a three-dimentional rigid structure. The former is β(1-4)glycan composed of alternating *N*-acetylglucosamine (GlcNAc) and *N*- acetylmuramic acid (MurNAc) whose carboxy group is point of linkage to the peptide. We previously demonstrated that the minimum structure required for the immunostimulation is *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipetide: MDP). Recent studies have shown that MDP acts via a Toll like receptor 2 (TLR2) independent pathway while TLR2 was described as the

receptor for PGN. Recently, intracellular protein Nod1 and Nod2 were discovered which were originally identified as a receptor against bacterial immunostimulating glycoconjugate lipopolysaccharide (LPS). Recent studies, however, have suggested that the Nod1 and Nod2 stimulatory component is not LPS, and is probably a biologically active contaminant in the LPS preparation. Nod1 and Nod2-stimulatory fraction was separated from the LPS fraction by gel-filtration column chromatography, and amino acid analysis indicated that its fraction contained PGN. Therefore, we have synthesized partial structures of PGN in order to investigate the structure recognized by Nod1 and Nod2. Partial structures of PGN such as MDP, MurNAc linked to L-Ala-D-isoGln-L-Lys (muramyl tripeptide), MurNAc linked to L-Ala-D-isoGln-L-Lys-D-Ala (muramyl tetrapeptide), $(GluNAc\text{-}MurNAc)_2$ linked to L-Ala-D-isoGln (tetrasacchride dipeptide) and $(GluNAc\text{-}MurNAc)_4$ linked to L-Ala-D-isoGln (octasaccharide dipeptide) were synthesized and their biological activities were measured. These glycopeptides all activate Nod2, where MDP showed the strongest activity. We found that Nod2 is a intracellular receptor for MDP as well as PGN. On the other hand, PGN of gram-negative bacteria was thought to be the ligand candidate for Nod1. PGN containing *meso*-diaminopimelic acid is uniquely present in certain bacteria including Gram-negative bacteria and particular Gram-positive bacteria. We thus synthesized several partial structures of PGN containing diaminopimelic acid such as L-alanyl-D-isoglutaminyl-diaminopimelic acid (A-iQ-DAP), D-isoglutaminyl-diaminopimelic acid (iQ-DAP) and γ-D-glutamyl-diaminopimelic acid (iE-DAP), and their biological activities were measured. These three peptides all activated Nod1, where iE-DAP showed the strongest activity. iE-DAP was hence identified as the minimal ligand for Nod1.

(521) **Synthetic Study on Microbial Peptidoglycan for Elucidation of TLR2 ligands**

Osamu Kubo[1], Seiichi Inamura[1], Zenyu Shiokawa[1], Artur J. Ulmer[2], Holger Heine[2], Yukari Fujimoto[1], Koichi Fukase[1] and Shoichi Kusumoto[1]

[1] Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan, [2] Department of Immunology and Cell Biology, Research Center Borstel 23845, Borstel, Germany.

Microbial peptidoglycan (PGN) , which is the component substance of bacterial cell wall, is well known as a potent immunostimulator. It is considered that toll-like receptor 2 (TLR2) is responsible to recognize the PGN and to activate the immunosystem. PGN consists of polysaccharide chains of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), linked to a peptide network to form a three dimensional rigid structure. We have previously demonstrated that *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide : MDP) is the immunostimulating minimal structure of PGN. However MDP was not recognized by TLR2, and TLR2 seems to recognize larger parts of PGN. We thus synthesized various partial structures of PGN including di-, tetra-, and octasaccharide having various peptides. However they are not recognized by TLR2, and we found an intracellular receptor NOD2 recognizes the MDP moiety as well as these PGN partial structures. In order to identify the structural motif of PGN recognized by TLR2, we have synthesized partial structures of peptidoglycan *(Staphylococcus aureus)* consisting of oligosaccharides (disaccharides) interlinked with peptides, expecting to form a certain conformation or lipophilicity with the penta glycine moiety. The disaccharides, GlcNAc(β1-4)MurNAc was prepared by stereoselective glycosylation of an *N*-acetyl muramic acid acceptor with *N*-troc glucosaminyl trichloroacetimidate using TMSOTf as the promoter. The peptide part was synthesized by solid-phase synthesis. For the peptide synthesis, first Npys-L-Lys(Fmoc)-OH was supported on the Wang resin with 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) and *N*-methylimidazole (NMI). Then Fmoc group of the Lysine on the resin was removed and Fmoc amino acids were condensed with *N*,*N*'-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) in sequence. After the construction of branched dodecapeptide chain, two disaccharides were introduced to the two amino groups of the peptide chain supported on the resin. The product was cleaved from the resin by treatment with TFA, and subsequent cleavage of the protecting group of the disaccharides gave the branched peptidoglycan partial structure. The biological activity will be investigated to characterize the structural motif responsible for TLR2 dependent pathway.

(522) **New Separation Method for (R)- and (S)-form of Lipopolysaccharide**

Takashi Goi, Shoichi Kusumoto and Koichi Fukase

Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan.

Lipopolysaccharide (LPS), a cell surface glycoconjugate of Gram-negative bacteria, shows both beneficial and adverse characteristics such as antitumor activity and lethal toxicity. LPS consists of three chemically different regions: the *O*-specific polysaccharide chain, the core saccharide, and lipid A component. Mature LPS of many wild-type bacteria contains a long polysaccharide chain. This type of LPS is called smooth (S)-form LPS. Mutant bacteria, which are completely defective in polysaccharide biosynthesis, produce LPS composed of the core saccharide and lipid A region. This type of LPS is named rough (R)-form LPS. Recently, it has been pointed out the mechanism of activation of macrophages or macrophages-like cells by S-form LPS may be different from that of R-form LPS. A separation of S-form LPS is therefore required for the precise characterization of biological differences between both forms of LPS. Several methods for separation of S- and R- species have been reported so far using gel permeation, hydrophobic chromatography or centrifugal partition chromatography (CPC). In the present study, we developed very simple separation method which can be used for separation of large amount of LPS. We previously identified branched peptidic structures (=molecular forceps) that recognized lipid A from combinatorial libraries. The consensus motif in the molecular forceps was branched tripeptide composed of three lysine residue, i.e., -Lys(Lys)-Lys-. We thought this motif should recognize phosphate groups in lipid A. The polymer support having this motif was expected to absorb lipid A via interaction between the branched tri-lysine and phosphate group. We also expected that more hydrophobic R-form LPS must be more easily adsorbed on amphiphilic polystyrene polyethylene glycol (PS-PEG) resin than S-form LPS because of the hydrophobicity of the resin. We therefore prepared the branched tri-lysine on amphiphilic PS-PEG resin. In fact, we successfully separated S-form LPS from LPS of *Escherichia coli Serotype 0111:B4* by simple procedure using this resin. The mixture of aqueous LPS solution and the resin beads having the molecular forcep was shaken slowly overnight. After confirming that R-form LPS were adsorbed on the beads by electrophoresis, the beads were removed by filtration. The filtrate was then lyophilized to give S-form LPS.

(523) **New Method for Deprotection of 2,2,2-Trichloroethoxycarbonyl (Troc) Group by Using Radical Reaction**

Hiroomi Tokimoto and Koichi Fukase

Depertment of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan.

2,2,2-Trichloroethoxycarbonyl (Troc) group has been frequently used for protection of amino and hydroxyl groups in organic synthesis. Deprotection of Troc group was generally carried out by using Zn/AcOH or Zn-Cu/AcOH. In the present study, we developed a new method for deprotection of *N*-Troc group by using radical reaction under microwave irradiation. We speculated that Troc group is removed by Zn/AcOH via a radical intermediate formed by abstraction of chlorine atom. We hence expected that other radical generating reagents can cleave Troc group via abstraction of chlorine. We first examined the reaction by using *N*-Troc glucosamine and the combination of AIBN and Bu_3SnH as a radical source in benzene. Troc group was partially removed as we expected but several byproducts were also formed. Since one of the byproducts should be dichloroethoxycarbonylated product formed by the reduction, we then examined the use of $(Bu_3Sn)_2$. Deprotection of Troc group proceeded slowly in benzene even under microwave irradiation. The reaction was not completed after 6 hours irradiation but the byproduct formation was reduced by using $(Bu_3Sn)_2$. The resulting amino group was then acetylated for easy separation using preparative TLC. The acetylated product was thus obtained in 46% yield. We then changed reaction solvent to DMF which has higher absorptance of microwave than benzene. Indeed, the reaction proceeded smoothly and was finished within 20 minutes. The acetylated product was obtained in 84% yield after purification by preparative TLC. Since the removal of Troc group by this method is carried out under homogenous conditions, the present method may be applicable to solid-phase synthesis. Application of the present method for synthesis of various oligosaccharide will also be discussed.

(524) **Development of a Glycoproteomics Strategy Enabling Identification of Both Core Proteins and Attached Glycans**

Ko Hayama and Jun Hirabayashi

Glycostructure Analysis Team, Research Center for Glycoscience, National

Institute of Advanced Industrial Science and Technology, Tsukuba Central 6, 1-1-1, Higashi, Tsukuba, Ibaraki, Japan .

Recently, innovating proteomic procedures targeting glycoproteins have been developed in 2 independent groups. They differ in the ways by which glycoproteins/glycopeptides are collected: Zhang et al. utilized hydrazide beads to capture glycoproteins, the glycan moieties of which have *cis*-diol groups that are subjected to periodate oxidation to generate aldehyde groups [1]. On the other hand, Kaji et al. made use of lectins, i.e., carbohydrate-binding proteins, for selective recovery of glycopeptides generated by tryptic digestion [2]. In fact, the latter scheme is a modification of the "glyco-catch" method, originally developed by the present authors [3, 4]. Though these procedures enable a large-scale identification of glycoprotein genes by means of 2-dimensional liquid chromatography/mass spectrometry/mass spectrometry (2D-LC/MS/MS) together with precise determination of *N*-glycosylation sites, neither of them gives detailed information on the attached glycan structures, because the glycans are liberated as a mixed fraction of glycopeptides.

We propose an improved strategy for glycoproteomics, which enables identification of both core proteins and glycan structures: the scheme consists of 1) selection of glycopeptides by lectin-affinity chromatography, 2) separation of glycopeptides by HPLC, 3) liberation of *N*-glycans in the presence of $H_2{}^{18}O$, 4) solid-phase purification of glycans and deglycosylated peptides and 5) analysis of their structures by MALDI-MS. The proposed strategy was first validated with a model glycoprotein, asialofetuin, to identify 3 *N*-glycosylation site and complex type triantennary saccharidesAsialofetuin has 3 *N*-glycosylation sites, at asparagine residues 99, 156, and 176, to which triantennary complex type *N*-glycans are attached as the major glycoform. All of 3 deglycosylated peptides were identified by MS spectra, whereas each of the glycan fractions, which were recovered by carbon graphite resin, gave a peak corresponding to NA3. As a more complex sample, soluble glycoproteins extracted from mouse liver and purified by ConA-agarose chromatography were also also analyzed. As a result, several glycopeptides were successfully characterized; i.e., identification of core proteins, glycosylation sites and glycan structures (in this case, high-mannose type). Thus, the improved strategy proved to be valid for more comprehensive analysis of glycoproteins from the scope of glycoproteomics.

This work is partly supported by the New Energy and Industrial Technology Development Organization (NEDO) in Japan.

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(525) DNA Microarray Analysis of Genes Responsible for the Expression of Carbohydrate Epitopes.

Hiromu Takematsu[1], Harumi Yamamoto[1,2], Yasushi Okuno[3], Reiji Kannagi[4], Akemi Suzuki[2] and Yasunori Kozutsumi[1,2]
[1] Graduate School of Biostudies, Kyoto University, Kyoto, 606-8501, Japan, [2] Supra-biomolecular System Group, RIKEN Frontier Research System, Wako 351-0198, Japan, [3] Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, 606-8501, Japan, [4] Department of Molecular Pathology, Aichi Cancer Center, Nagoya 464-8681, Japan.

Changes in the expression of glyco-chains have influence on many biological events, such as embryogenesis, oncogenesis, lymphocyte activation, differentiation and others. Since glyco-chains are biosynthesized by tandem glycosyltransferase reactions in Golgi apparatus, change in any reaction could alter the final product in the biosynthetic pathway of glyco-chain, including sugar metabolism, nucleotide-sugar transport, glycosyltransferase and glycosidase. In fact, the gene/enzyme identified through 'loss of function'-based strategy should contribute the the biosynthesis of the glyco-chain, there could be other gene/enzyme which actually regulate the production of the glyco-chain. To understand the biological role and mechanism of the regulation of the glyco-chain expression, systematic studies on the overall regulation of gene expression are important. For this purpose, based on the efforts of the community in decades for the cloning of genes for glyco-chain biosynthesis, we made a DNA microarray and expression of a large number of genes was monitored at once to. The DNA microarray is a cDNA-type array with 300bp length probes in average and has about 1000 genes of glycosyltransferases, glycosidases, nucleotide sugar-modification enzymes and other components related to carbohydrates. These genes cover glycoproteins, proteoglycans, glycosphingolipids and sphingolipids. As a model, gene expression related to carbohydrate epitope expression of human cell lines was analyzed by the microarray using a mRNA mixture prepared from human organs as an internal standard. Concomitantly, these cell lines were stained with carbohydrate-specific monoclonal antibodies and analyzed by FACS. When these data were compared, a good correlation was found between the expression profile of genes and the staining profile of the monoclonal antibodies. As the second example, the carbohydrate epitope of a monoclonal antibody specific to activated B cells was determined by the same system. These data suggest that DNA microarrays are powerful tools for the analysis on gene expression involved in regulation of the glyco-chain expression.

(526) Synthesis and Biological Activity of *Helicobacter pylori* Lipopolysaccharide Partial Structures Containing 2-keto-3-deoxy-D-manno-octonic acid (Kdo)

Masato Iwata[1], Noriko Imakita[1], Yasuo Suda[2], Yukari Fujimoto[1], Koichi Fukase[1] and Shoichi Kusumoto[1]
[1] Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan, [2] Graduate School of Science and Engineering, Kagoshima University, Kagoshima 890-0065, Japan.

Helicobacter pylori is Gram-negative bacteria often observed in the stomach of patients with chronic gastritis. The bacteria are also considered to be a possible cause of gastric and duodenal ulcers. *H. pylori* lipopolysaccharide (LPS) shows very low endotoxic activity in comparison to other enterobacterial preparations but definite IL-6 and TNF-α inducing activity. *H. pylori* LPS has the distinct chemical structure in lipid A part from those of the enterobacterial LPS. The chemical structure of *H. pylori* lipid A is different from that of *Escherichia coli* in the following points; (1) the presence of less number but of longer fatty acid residues; (2) the absence of the 4-phosphate group; and (3) the presence of an ethanolamine group non-stoichiometrically linked to the glycosyl phosphate functionality. We have so far synthesized *H. pylori* lipid A and found that synthetic lipid A showed identical biological activities in induction of IL-18 with the natural *H. pylori* LPS but no activities in induction of IL-6 and TNF-α. We previously found that 3-deoxy-D-manno-2-octurosonic acid (Kdo) residues existing in liker region of lipid A and polysaccharide part increase the cytokine inducing activity by the chemical synthesis of LPS partial structures consisting in lipid A and Kdo. In this study, we synthesized *H. pylori* lipid A analogue containing Kdo to investigate the effect of Kdo residue in the biological activity, especially on the IL-6 and TNF-α induction. The β(1→6)-disaccharide structure of lipid A was constructed by the glycosylation of a 6-OH-*N*-acyl glucosamine acceptor with an appropriately protected 2-*N*-Troc-1-*O*-trichloroacetimidate donor. The 6'-position of the lipid A was then converted to the triethylsilyl (TES) group, and the glycosylation with Kdo fluoride afforded α-Kdo glycoside. The selective phosphorylation at the 1- position and cleavage of the protecting group gave Kdo-*H. pylori* lipid A. The biological activity of the Kdo-*H. pylori* lipid A will be also discussed.

(527) Synthesis and Biological Activities of Lipid A Mimics Possessing Acidic Amino Acid

Masao Akamatsu[1], Mikayo Kataoka[1], Yukari Fujimoto[1], Yasuo Suda[2], Koichi Fukase[1] and Shoichi Kusumoto[1]
[1] Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan, [2] Graduate School of Science and Engineering, Kagoshima University, Kagoshima 890-0065, Japan.

Lipopolysaccharide (LPS), cell surface glycoconjugate of gram-negative bacteria, has been known as a potent immunostimulator. The active principle of LPS is "lipid A", which induces various cytokines to activate the immune system. Lipid A is an amphiphilic molecule composed of hydrophilic glucosamine disaccharide part with two phosphate groups and hydrophobic acyl chains. We have synthesized a series of lipid A analogues and shown that two acidic groups and acyl chains are crucial for the activity of lipid A. The hexa-acylated *E. coli* lipid A shows potent immunostimulating activity, whereas tetra-acylated lipid A (biosynthetic precursor) and tri-acylated lipid A have antagonistic activity against LPS. As for the role of phosphate groups, *E. coli* lipid A analogues lacking 1- or 4-phosphate showed considerably weaker activity than lipid A, and a lipid

A analogue lacking both phosphates did not show the activity. Lipid A analogues having a carboxymethyl (CM) group at the 1-position in place of the phosphate exhibited biological activity indistinguishable with that of lipid A.

We thought spatial arrangement of two acidic functional groups but not glucosamine disaccharide structure is important for the activity. In the present study, we hence designed lipid A analogues where non-reducing end glucosamine structure was replaced by acidic amino acids, i.e., hosphoserine or aspartic acid, based on molecular mechanics calculation.

The target compounds possessed 1-*O*-carboxymethyl group and either L- or D-amino acids at 6-position of glucosamine. They had three types of acylation patterns as follows; 3-hydroxytetradecanoyl group at 2-*N*-, 3-*O*-, and α-*N*-positions (tri-acyl type), tetradecanoyloxytetradecanoyl group at α-*N*-position and 3-hydroxytetradecanoyl group at 2-*N*- and 3-*O*-positions (tetra-acyl type I), and 3-hydroxytetradecanoyl group at 2-*N*-, 3-*O*-, 4-*O*-, and α-*N*-positions (tetra-acyl type II).

The synthesis was carried out as follows. The protected amino acids were introduced to 6-position of a common monosaccharide intermediate. Acyl groups were introduced at the respective positions step by step to afford a library of 12 compounds.

Cytokine induction assay showed that most of the analogues have antagonistic activity against LPS. The tetra-acyl type I analogues showed the strongest activity and the tetra-acyl type II analogues showed somehow weaker activity. The activities of the tri-acyl type analogues were much weaker. Interestingly, only tetra-acyl type I analogue having D-aspartic acid residue showed weak but definite cytokine induction. As for *Limulus* activity, tetra-acyl type I analogues showed highest activity and tri-acyl type analogues showed weaker activity, whereas tetra-acyl type II analogues did not show *Limulus* activity. Stereochemistry of amino acid residue did not largely influence the activities.

Conformations of the synthetic analogues were estimated by molecular mechanics calculation and the result will be discussed in relation to their biological activities.

(528) **Biochemical Characterization of Myoblasts from Hereditary Inclusion Body Myopathy Patients with Various Mutations in GNE**

Ilan Salama[1], Stephan Hinderlich[2], Zipora Shlomai[3], Iris Eisenberg[1], Sabine Krause[4], Kevin Yarema[5], Zohar Argov[6], Werner Reutter[2], Hanns Lochmuller[4], Ron Dabby[7], Menachem Sadeh[7], Hannah Ben-Bassat[3] and Stella Mitrani-Rosenbaum[1]

[1] Goldyne Savad Institute for Gene Therapy, Hadassah-Hebrew University Medical Center, Jerusalem, Israel , [2] Institut fur Molekularbiologie und Biochemie, CharitÈ - Universit‰tsmedizin Berlin, Berlin-Dahlem, Germany, [3] Laboratory of Experimental Surgery , Hadassah-Hebrew University Medical Center, Jerusalem, Israel, [4] Friedrich-Baur-Institut und Genzentrum, Ludwig-Maximilians-Universit‰t M¸nchen, Munich, Germany, [5] Whitaker Institute of Biomedical Engineering, The Johns Hopkins University, Baltimore, USA, [6] Department of Neurology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel, [7] Department of Neurology, Wolfson Hospital, Holon, Israel.

Hereditary inclusion body myopathy (HIBM) is a unique group of neuromuscular disorders characterized by adult-onset, slowly progressive distal and proximal muscle weakness, which is caused by mutations in UDP-N-acetylglucosamine 2-epimerase/ N-acetylmannosamine kinase (GNE), the key enzyme in the biosynthetic pathway of sialic acid. GNE consists of two functional domains, an epimerase domain and a kinase domain. In contrast to the Persian Jewish and other Middle Eastern patients which present a homozygous mutation in the kinase domain of GNE, most of the HIBM affected individuals of various ethnicities are compound heterozygotes, where all combinations of mutations among the domains, epimerase-epimerase, kinase-kinase and epimerase-kinase, were found. Previously we have shown that the reduced GNE enzymatic activity in lymphoblastoid cell lines of HIBM patients does not affect the overall sialylation in these cells. In order to investigate the consequences of the mutated GNE enzyme in muscle cells, we established cell cultures from muscle biopsies from 11 HIBM patients (9 patients carrying the Middle Eastern kinase homozygous mutation, one patient carrying an epimerase homozygous mutation and one compound heterozygote patient with one mutation in each domain) and from 9 controls. These muscle cultures were examined for their epimerase activity and membrane bound sialic acid content. Myoblasts carrying the homozygous kinase mutation (M712T) presented a 30% reduction in epimerase activity. This activity was reduced to 50% in cells from the compound heterozygote patient as well as the patient carrying the homozygous epimerase mutation. Membrane bound sialic acid measurements showed that patient-derived myoblasts, carrying either the kinase/kinase or the epimerase/kinase mutations, exhibit approximately 80% of the bound sialic acid amount compared with controls, while cells with the epimerase/epimerase mutations in GNE had a 60% reduction. No differences were observed in the amount of sialic acid on membrane N- and O-linked glycoproteins either by Western blot or by flow cytometry in any of the mutants analyzed. These results show that in spite of the feed back inhibition mechanism of CMP-sialic acid on GNE epimerase activity, reduced enzyme activities of mutated GNE may decrease the amount of sialic acids in muscle cells. However the lack of correlation between the amount of sialic acid in muscle and the HIBM phenotype may indicate that the pathological mechanism of the disease may not be linked to the well characterized sialic acid pathway.

(529) **Biological Potential of 6-Sulfo-Galactosamines and Their Polyvalent Models as Homologues of *N*-Acetyl-Neuraminic Acids**

Mikie Kambara[1], Kenji Sasaki[1], Yoshihiro Nishida[1], Hirotaka Uzawa[2] and Kazukiyo Kobayashi[1]

[1] Department of Molecular Design and Engineering, Graduate School of Engineering, Nagoya University, Chikusa-ku, Nagoya 464-8603, Japan, [2] Research Center of Advanced Bionics, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba 305-8565, Japan.

In our preceding study, we encountered the fact that the polyvalent 6-sulfo-GlcNAc derived by radical polymerization show a notable activity of blocking L-selectin (*Angew. Chem. Int. Ed.*, *41*, 4463, **2002**). The result strongly suggested that the 6-sulfo-GlcNAc might possess high biological potential as a structural homologue of *N*-acetyl-neuraminic acid (NeuAc). With NMR and MM calculations and in vitro biological assays using neuraminidases, we have collected evidence for structural homology between 6-sulfo-GlcNAc and NeuAc (*Bioorg. Med. Chem. Lett.*, *13*, 2821, **2003**: *Bioorg. Med. Chem.*, *12*, 1367, **2004**). In the present study, we speculated that 6-sulfo-galactosamine (6-sulfo-GalNAc) would serve as a better NeuAc homologue than the 6-sulfo-GlcNAc. This is partly because the axial OH-4 group has a stereochemical effect on locating the 6-sulfate group in geometry close to NeuAc-2-ene. This suggests that the 6-sulfo-GalNAc derivatives may serve as neuraminidase inhibitors. Moreover, the 6-sulfo-GalNAc are expected to mimic NeuAc-(2-6)-Gal linkages, suggesting that they may serve also as hemagglutinin blockers. The diverse biological activities may lead to the development of anti-Influenza reagents. Å@To evaluate the above working hypothesis, a series of 3-sulfo, 4-sulfo, and 6-sulfo-GalNAc derivatives were prepared as *p*-nitrophenyl (*p*NP) β-$_D$-glycosides, some of which were converted also to polyvalent models (acrylamide copolymers). The high biological potential of the 6-sulfo-GalNAc was evidenced by the *in vitro* biological assays using an Influenza neuraminidase. The inhibitory activity of *p*NP 6-sulfo-GalNAc was ca. 100 folds as potent as that of *p*NP 6-sulfo-GlcNAc. The polyvalent 6-sulfo-GalNAc showed notable blocking activity to Influenza hemagglutinins, while the polyvalent 6-sulfo-GlcNAc showed little activity. Obviously, the skeleton of the 6-sulfo-GalNAc mimics both of NeuAc-2-ene and NeuAc-(2-6)-Gal linkages.

(530) **Pan-Selectin Glycomimetic Inhibitors Inhibit Rolling and Neutrophil Migration in Mouse Models of Inflammation**

Theodore Smith[1], Mauro Perretti[2], John T. Patton[1], Arun Sarkar[1] and John L. Magnani[1]

[1] GlycoMimetics Inc., 101 Orchard Ridge Dr., Suite 1E, Gaithersburg, MD 20878 USA, [2] The William Harvey Institute, London, UK.

Heterobifunctional glycomimetic compounds designed to interact with both carbohydrate and sulfate-binding domains of the selectins are potent inhibitors of E, P and L-selectins. Two of these compounds, GMI-1010 and GMI-1011, were tested in mouse models of inflammation. The first model tested the effect of the glycomimetic compounds on leukocyte rolling during a fulminating inflammatory response by intravital microscopy. Normal male Swiss albino mice were injected intraperitoneally with IL-1B to induce an inflammatory response. After 4 hours, at the height of increased leukocyte rolling, GMI-1010 and GMI-1011 were administered through the jugular vein. Rolling of leukocytes was immediately inhibited and the degree of inhibition progressed throughout the entire observation period of 30 minutes ending with an inhibition of 80% of cell flux. The second model tested the effects of GMI-1010 and GMI-1011 on the inhibition of leukocyte migration into air pouches formed on the backs of normal male Swiss albino mice by air injection. Six days after formation of

the air pouches, an inflammatory response was initiated by injection of IL-1B. GMI-1010 and GMI-1011 were administered either at time zero, 2 hours after injection, or dosed at both time zero and 2 hours. Air pouches were then lavaged 4 hours after stimulation and the number of leukocytes migrated into the pouch were counted. In all cases GMI-1010 and GMI-1011 inhibited neutrophil migration into the pouch. GMI-1011 was consistently more active and displayed inhibitory activity at 2mg/kg and significant inhibition at 5 mg/kg. These heterobifunctional pan-selectin inhibitors demonstrate strong activity over a relatively long time period of time in vivo and represent a new class of selectin inhibitors for the development of therapeutics for inflammatory diseases.

(531) **Development of a Lectin Microarray for the Glycomic Profiling of Cells**

Lara K. Mahal, Kanoelani Pilobello and Lakshmi Krishnamoorthy
University of Texas at Austin, Chemistry and Biochemistry Department, 1 University Station, A5300, Austin, Texas 78712.

Glycosylation is involved in a diverse array of biological processes which include tumor cell metastasis, inflammation and cellular signaling. Unfortunately, the systematic study of glycosylation is complicated by the lack of rapid, easily available techniques for the survey of cell surface glycans. This dearth of information has also prevented application of bioinformatics to the study of glycosylation patterns. The advent of microarray technology has opened the door for rapid characterization of complex mixtures of proteins or DNA. This paper describes the development of a lectin-based microarray for the rapid profiling of cellular carbohydrates (glycomics).

(532) **Capture and Release of Proteins by Stimuli-Responsive Glyco-Polypeptide**

Xue-Long Sun[1] and Elliot L. Chaikof[1,2]
[1] Department of Surgery and Biomedical Engineering, Emory University School of Medicine, Atlanta, Georgia 30322 , [2] School of Chemical Engineering, Georgia Institute of Technology, Atlanta, Georgia 30332.

Cell surface carbohydrates act as receptors for a variety of protein ligands and thereby provide a starting point for the development of novel diagnostic agents. From this point, we have designed and synthesized a thermal-responsive glyco-polypeptide by recombinant and chemical techniques for protein capture and release application. The ideal biocapture potentiality of the glycoconjugate is facilitated by combining with its biomimetic glycoconjugate, multivalent carbohydrate-protein interaction and thermal-responsive properties. Especially, both capture and release processes are conducted in solution status which will improve their efficiency highly. This platform opens future possibilities for protein or cell isolation and pathogen detection by variation of the bound carbohydrate.

(533) **Quantitative Carbohydrate Analysis: Results of a Multi-Laboratory Benchmarking Exercise.**

Joseph W Siemiatkoski.
14 Cambridge Center, Cambridge, MA 02142.

Samples of an immunoglobulin class G (IgG) and a mammalian serum glycoprotein were distributed to laboratories engaged in the performance of glycoprotein analyses. The aim of this exercise was to provide a basis for improvement of the overall quality of glycoanalytics, and to evaluate the performance of the various methods used to determine glycoprotein heterogeneity. The samples were analyzed for glycan distribution by each laboratory using their own methods. Glycan distribution results obtained by the participating laboratories will be presented as participant-blinded results, and will be compared and contrasted based on the analytical methods employed.

(534) **A Cell-Based Assay for the Evaluation of Sialyltransferase Inhibitors**

Karin Norgard-Sumnicht and James C. Paulson
The Scripps Research Institute,
Departments of Molecular Biology and Molecular and Experimental Medicine,
La Jolla, California 92037.

Glycosyltransferases are gaining increasing attention for their potential as therapeutic agents aimed at modulating protein carbohydrate interactions that are involved in immune regulation.(1, 2) Glycosyltransferases reside in the organelles of the secretory pathway requiring that an inhibitor cross two membranes. Thus, an important step to bringing potential inhibitors to animal model studies is evaluation of their ability to inhibit their target *in situ*. The most convenient way to detect the effect of the inhibitor in a cell-based assay is to assess the change in glycosylation at the cell surface. For this purpose there are many carbohydrate specific lectins and antibodies that can be used to detect such changes. The problem, however, is that cell surface glycans are stable and turnover slowly, thus the effect of the inhibitor may take days to be revealed.

In order to develop a convenient cell-based assay for the ST6Gal I sialyltransferase, we have used a naturally-occurring CHO K1 mutant cell line, the ldlD CHO first described by Krieger and colleagues.(3, 4) These cells are deficient in the UDP-Glc(NAc)-4-epimerase and are therefore unable to produce either UDP-Gal or UDP-GalNAc from their respective precursors. The result is that under growth conditions without Gal or GalNAc, fully elongated carbohydrate structures are not produced and no terminal sialic acids are detectable. Upon addition of Gal and/or GalNAc to the media the cells quickly uptake these monosaccarides and produce fully elongated and sialylated structures within hours. Thus, in principle, an inhibitor can be added to cells containing no sialic acid prior to initiating sialoside synthesis by addition of Gal and/or GalNAc.

Since CHO K1 cells normally only produce α2,3-linked sialic acids, we transfected the human ST6Gal I enzyme into these cells, and created a stable cell line capable of displaying both α2,3 and α2,6-linked sialic acids. Using *Sambucas nigra* and *Mackia amurensis* II lectin staining to detect α2,6 and α2,3-linked sialic acids by FACS analysis, respectively, we demonstrate the utility of this assay system and show that we can distinguish between *N*- and *O*-linked structures affected. This system appears to be well suited for evaluating sialyltransferase inhibitors *in situ*.

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(535) **Development of Glycoarray having the Functionalized Oligosaccharides toward the Chemical Genomics**

Daisuke Takahashi, Hiroshi Tanaka and Takashi Takahashi
Department of Applied Chemistry, Graduate School of Science and Engineering, Tokyo Institute of Technology, 2-12-1 Ookayama, Meguro, Tokyo 152-8552, Japan.

Carbohydrates play a central role in living organisms as recognition markers to enable cell adhesion, fertilization, differentiation, development, and tumor-cell metastasis through carbohydrate-protein (lectin) interactions. Human genome project has provided information of protein sequence involving the lectins. Additionally, recent development of combinatorial chemistry allows for the rapid assembly of carbohydrate libraries. Therefore, analysis of the carbohydrate libraries-proteins interaction should give us important information related to the biological events on cell surface. However, the carbohydrate-protein interactions are weak in comparison with protein-protein or DNA-protein interactions. Therefore, an effective analytical methodology for high through-put analysis of carbohydrate-protein interaction is required.

In the last decade, biological microchips (biochips) such as DNA and protein chips have been fabricated for a variety of applications. Carbohydrate-based arrays would be an effective tool for the high-throughput study of carbohydrate-protein interactions. However, the synthesis of carbohydrate-based array to detect the week interaction would be an challenging task. Herein we report the development of the efficient reaction to immobilize carbohydrate probes on the surface and investigation of the suitable conditions in a protein-binding assay.

Our strategy for the synthesis of glycoarray involves S-alkylation of thiol-containing carbohydrate probes with α-bromo acetamide on glass slide. S-alkylation of α-bromo acetamide with thiols would smoothly proceed under the mild and aqueous conditions. Additionally, the remaining α-bromo acetamide would show relatively low non-specific adhesion of proteins. The α-bromo acetamide on glass slides would be prepared by acylation of amino-coated glass slides.

The synthesis of microarray attached with mannosides was investigated. Validating the mannoside slides can be achieved using fluorophore-labeled Concanavalin A (ConA). To investigate of the suitable immobilization conditions, we printed solutions of mannoses having two different spacers at the reducing end under various reaction conditions varying four

concentrations (10, 1.0, 0.1, and 0.01 mM), four pH, and reaction time (18, 6.0, 3.0, 1.0 h) on the three different coated slides. Binding of each spot to fluorophore-labeled Concanavalin A was tested. The slides were treated with a solution of bovine serum albumin (BSA) in PBS buffer that contained Tween 20 to reduce background fluorescence caused by nonspecific binding of fluorophore-labeled protein. Subsequently, the microspotted slides were probed with Concanavalin A (200, 20, 2.0 μg/mL) for 1 h. After extensive washing of the lectin-treated slides with the same buffer, the fluorescence intensity of microspots on the slides were determined by using a laser scanner.

Spots on the MAS coated glass slides were clearly detectable by the labeled lectin. Longer spacer was effective for binding to proteins than the shorter one. Loading reactions using 10 mM solution of mannoside under neutral conditions would proceed well. On the other hand, the microspots connected Galactose probe did not show any fluorescent signal.

In summary, we have developed a microarry attaching to carbohydrates through a covalent bond. Lengths of spacer and the concentration of the carbohydrate ligands are important for strong binding to lectins. Based on the observation, the synthesis of glycoarray attaching to various oligosaccharides is in progress.

(536) **Prediction of Glycan Structures from DNA Microarray Data**

Shin Kawano, Kosuke Hashimoto, Takashi Miyama, Susumu Goto and Minoru Kanehisa

Bioinformatics Center, Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan.

While the amino acid sequence of proteins is determined by the genetic code and the template in the genome, the carbohydrate sequence of glycans is determined by the biosynthetic code, a specific set of biosynthetic reactions catalyzed by different types of glycosyltransferases. Each glycosyltransferase catalyzes formation of a glycosidic-bond between the glycan precursor as an accepter and the nucleotide-activated sugar as a donor. Thus, once we know the repertoire of glycosyltransferases in the genome or in the transcriptome, as well as the repertoire of glycosidases and other sugar-related enzymes and the supply of donor monosaccharides, it should in principle be possible to predict the repertoire of possible glycan structures in an organism or at a specific stage of the cell. Here, we construct a reaction pattern library consisting of bond-formation patterns of glycosyltransferase reactions. Using the KEGG/GLYCAN structure database and the library, we try to predict the repertoire of possible glycan structures from microarray expression data for human genes. In order to construct the glycosyltransferase reaction pattern library, human glycosyltransferase genes were collected from the KEGG/GENES database based on its annotations. The reaction specificity was determined according to the published literature and was characterized by the following three features: 1) the acceptor monosaccharide in the glycan chain, 2) the donor monosaccharide and 3) the glycosidic-bond pattern between them. We collected 146 glycosyltransferase genes in the human genome and classified them into 41 donor-acceptor pairs. The library contained 9 kinds of monosaccharides: glucose, galactose, mannose, N-acetyl glucosamine, N-acetyl galactosamine, fucose, xylose, glucuronic acid and N-acetyl neuraminic acid. Human glycan structures were extracted from the KEGG/GLYCAN database, which contained glycans from various organisms. The entries only with those 9 monosaccharides present in the library were extracted as human glycans. As of June 2004 there are 10,386 entries (unique glycan structures) in the KEGG/GLYCAN database, and 4,198 human glycan entries were extracted. The prediction of glycan structures from microarray expression data is performed as follows. When the expression level of a gene in a given sample changed compared to the control sample, the corresponding glycosyltransferase was searched against the pattern library and the donor-acceptor pair was identified. These pairs were searched against the human portion of the KEGG/GLYCAN database using KCaM, which is a database search program for similar glycan structures. We will report examples of specific glycan structures predicted by this method using publicly available microarray data.

(537) **Application of Permethylation/Ion Trap MS^n/Fragmentation Library Strategies to Glycosphingolipid Structure Elucidation**

Suddham Singh, Beau Bennion, Vernon N. Reinhold and Steven B. Levery

Department of Chemistry, University of New Hampshire, G229 Parsons Hall, Durham, NH 03824-3598, USA.

Glycosphingolipids (GSLs) are essential components of the cell membranes of all eukaryotes and some bacteria. GSLs are highly varied in their structures and resulting physico-chemical properties, their cell/tissue/developmental/species distribution patterns, and their functional roles (which are not well understood). "Glycosphingolipidomics" thus represents an important subdivision of both glycoconjugate and lipid metabolomics, for which rapid, broadly applicable analytical strategies have not yet been established. The great complexity and variability of GSL structures and their expression patterns calls for development of multi-purpose methodologies which are well-suited for sensitive, accurate, high throughput profiling of GSL expression, yet also adaptable for applications where detailed characterization of individual GSL components, particularly with respect to the primary structure of the head groups, is necessary.

For this study, ion trap MS^n/fragmentation library methodologies were applied to GSLs from a variety of sources, including glycosylceramides from High Five™ cultured insect cells and glycosylinositol phosphorylceramides (GIPCs) from fungi. Comparison of MS^n spectra of a variety of di- and trisaccharides from GSLs of known structure indicated that clear and reproducible differences in fragmentation could be observed as a function of differences in anomeric configuration as well as linkage position. Interestingly, MS^{n+1} spectra arising from identical structures generated in situ from different precursor ions via alternate MS^n pathways were compared and observed to be similar, suggesting identical intermediate MS^{n+1} precursor ions are isoenergetic regardless of ultimate origin. Development of an MS^n library will aid in wider application of these techniques, and this library is being expanded to include glycosphingolipid glycans and glycosylinositols of increasing complexity. Experimental: Glycosylceramides were permethylated by the method of Ciukanu and Kerek [(1984) Carbohyd. Res. 131:209-217]. In the case of GIPCs from fungi, glycosylinositols were first freed from ceramide and phosphate by ammonolysis [Barr et al (1984) Biochemistry 23:5589-5596; Levery et al (1998) Biochemistry 37:8764-8775] (10 N aqueous NH_3 in sealed tube at 150∞C 12-16 h; isolation from lipid byproducts by passage through C18-silica SPE cartridge in H_2O), then permethylated. In addition, released glycosylinositols containing a 2-deoxy-2-amino-glucosamine residue were selectively N-acetylated (acetic anhydride/pyridine in MeOH) prior to permethylation. Ion trap MS^n analysis was performed on ThermoFinnigan LCQ and LTQ instruments. Samples dissolved in 1 mM aq. sodium acetate/MeOH (1:1 v/v) were introduced via direct infusion.

(538) **An economic Approach to Isotope Enrichment of Glycoproteins for NMR with Sf9 Cells and Baculovirus.**

Wendy J. Walton[1], Agnieszka J. Kasprzak[2] and Timothy M. Logan[1,2,3]

[1] Graduate Program in Molecular Biophysics, Florida State University Tallahassee, FL 32306, [2] Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL 32306, [3] National High Magnetic Field Laboratory, 1800 E. Paul Dirac Drive, Tallahassee, FL 32310.

We are interested in the three dimensional structures of intact glycoproteins and are developing the tools for determining high-resolution structures of intact glycoproteins by using multi-dimensional heteronuclear NMR. Our current research is focused on developing economic approaches to uniform isotopic enrichment of carbon and nitrogen atoms of the protein and carbohydrate components. Our strategy is to minimize the accumulation of toxic metabolites while optimizing recombinant protein expression. A key aspect of this work is to reduce the amount of glutamine in the cell culture medium while maintaining sufficient energy yielding metabolites for vigorous growth. We are also investigating methods for incorporating ^{13}C and ^{15}N isotopes into the carbohydrates. We present an analysis of cell growth and protein production in Sf9 insect cells expressing recombinant Thy1-GFP fusion construct. We also demonstrate the uniformity of isotopic enrichment by NMR spectroscopy.

(539) **Glycoprotein Molecular Probes for Functional Analyses of Protein Quality Control System**

Yukishige Ito.[1,2]

[1] RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama, 351-0198 Japan, [2] CREST (JST), Kawaguchi, 322-1102 Japan.

N-Glycosylation of Asn is a prominent modification of eucaryotic proteins. It occurs co-translationally in ER and then becomes a subject of further processing by a variety of glycosidases and glycosyltrasnferases to produce highly diverse structures. In spite of the advance in oligosaccahride synthesis technology, chemical synthesis of these glycoprotein oligosaccharides is still a substantial challenge. One of the major obstacles in the synthesis of Asn-linked oligosaccharide has been the formation of β-glycosidic linkage between core mannose (Man) and N-acetylglucosamine

(GlcNAc). We successfully found a solution of this problem using p-methoxybenzyl assisted intramolecular aglycon delivery (Ito et al. Synlett 1998, 1102-1104).With this methodology in hand, our interest is now directed to biological functions of Asn-linked oligosaccharides, with particular stress placed on their roles in protein quality control (Helenis and Aebi, Science, 2001, 291, 2364-2369). It is becoming clear that a number of intracellular proteins are involved in this process. For instance, endoplasmic reticulum (ER) residing molecular chaperones calnexin (CNX) and calreticulin (CRT) are considered to recognize the oligosaccharide portion (Glc1Man9GlcNAc2) of glycoproteins and assist their folding. Subsequently, terminal glucose (Glc) is removed by glucosidase II and glycoproteins carrying undecasaccharide (Man9GlcNAc2) are transported to Golgi for further processing. Other major players in glycoprotein quality control are glucosyl transferase (UGGT), mannosidase-like lectin (EDEM) and cargo receptors (VIP36, ERGIC-53) and ubiquitin ligase (Fbx2). All of these proteins likely recognize precisely different oligosaccharide structures, although molecular basis of these phenomena is unclear. As the first step to gain clear understanding of these processes, we achieved the first chemical synthesis of dodecasaccharide (Glc1Man9GlcNAc2), which is a putative ligand of CNX and CRT, by using stereoselective β-mannosylation as the key reaction. During its synthetic studies, the utility of high-pressure conditions for selective removal of protecting group from complex and hindered intermediates was discovered (Matsuo et. al. Tetrahedron Lett. 2002, 43, 3273-3275). Binding with molecular chaperone was examined using NMR, which revealed that carbohydrate recognition by CRT was specific to Glc1Man9GlcNAc2 having a-linked glucose at terminal (Matsuo et. al. J. Am. Chem. Soc. 2003, 125, 3402-3403). Using the similar strategy, glucose undecasaccharide (Man9GlcNAc2) and Man8 containing deca- (Man8GlcNAc2) and undecasacharide (Glc1Man8GlcNAc2) were also prepared (Matsuo et. al. Carbohydr. Res. 2003, 338, 2163-2168). Using synthetic Man9GlcNAc2, it was revealed that novel ubiquitin ligase fragment Fbx2 recognizes high-mannose type oligosaccharide (Yoshida et. al. Nature 2002, 418, 438-442). Currently, a systematic study is in progress to 1) comprehensively prepare N-linked glycans and library of their partial structures (for instance, Takatani et. al. Carbohydr. Res. 2003, 338, 1073-1081), that may exist in ER and play roles in protein quality control, and 2) synthesize glycoprotein having homogeneous glycan chain. These molecular probes will be used for interaction analyses with lectins/chaperones, using various means including NMR and isothermal titration calorimetry (ITC).

(540) **Structure Analysis of Invertebrate N-Glycans by Comparative Biochemical View**

Shunji Natsuka[1,2] and Sumihiro Hase[1]
[1] Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan, [2] CREST, JST, 4-1-8 Honcho Kawaguchi, Saitama, Japan.

We previously reported that *N*-glycans of the nematode, *Caenorhabditis elegans*, were very similar to those of insect (1). This observation harmonizes with a theory that nematode and insect are closely related in phylogeny, and belong to Ecdysozoa, which is one of the two categories of protostome. Then, what kinds of *N*-glycans are present in Lophotrochozoa, another protostome? In order to answer this question, we analyzed *N*-glycan structures of squid, *Tedarodes pacificus*, which belonged to Lophotrochozoa. The pyridylaminated *N*-glycans were prepared from squid skin by hydrazinolysis and re-*N*-acetylation followed by fluorescent tagging with 2-aminopyridine (2, 3). Pyridylaminated *N*-glycans were separated into neutral and acidic fractions by DEAE-HPLC. The neutral fraction contained most *N*-glycans. The neutral *N*-glycans were purified and their structures were analyzed by the two-dimensional HPLC mapping method combined with glycosidase digestions and mass spectrometry. As a result, we found that high mannose-type glycans and biantenna-type *N*-glycans consisted of the type-1 structure (Galβ1-3GlcNAc) were dominant in the squid skin, and pauci-mannose-type was next to them. The *N*-glycans containing type-2 structure (Galβ1-4GlcNAc) was not detected in squid skin. Fucose was bound to the chitobiose core mainly by the α1-6 linkage, and partly by the α1-3 linkage. Judging from these structural features, the *N*-glycan structures in Lophotrochozoa might be related to those in both deuterostome and Ecdysozoa. Isomeric structures of high-mannose type glycans were the same as those found in mammals and *C. elegans*. The finding that these isomeric structures were highly conserved in metazoan may suggest physiological significance of branched structures of high-mannose type glycans. References: (1) Natsuka, S., Adachi, J., Kawaguchi, M., Nakakita, S., Hase, S., Ichikawa, A., Ikura, K. *J. Biochem.*, 2002, 131, 807-813. (2) Hase, S., Ikenaka, T., Matsushima, Y. *Biochem. Biophys. Res. Commun.*, 1978, 85, 257-263. (3) Natsuka, S., and Hase, S. *Methods Mol. Biol.*, 1998, 76, 101-113.

(541) **Enzymatic Reconstruction of Glycosaminoglycans**

Keiichi Takagaki, Atsushi Kon and Ikuko Kakizaki
Biochemistry, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan.

Proteoglycans (PGs) are complex glycoconjugates that are composed of a core protein and glycosaminoglycan (GAG) chains. The GAG chains are covalently bound to the core protein through a Gal-Gal-Xyl-Ser linkage region. The GAGs are composed of repeating disaccharide units: uronic acid (glucuronic acid, GlcUA or iduronic acid, IdoUA) combined with hexosamine (*N*-acetylglucosamine, GlcNAc or *N*-acetylgalactosamine, GalNAc or glucosamine, GlcN), and then classified into chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, etc, by the specific uronic acid/hexosamine combination. The endo-type glycosidases were investigated with the aim of performing enzymatic synthesis of PG. It is known that many glycosidases catalyze transglycosylation reaction as a reverse reaction in addition to their main hydrolysis reaction. Therefore, the transglycosylation mechanism of testicular hyaluronidase, which is an endo-β- acetylhexosaminidase, was investigated. When hyaluronic acid (HA), longer than hexasaccharides, having a GlcUA at the nonreducing terminal was used as a substrate, this enzyme released disaccharides with a structure of GlcUAβ1-3GlcNAcÉ“1 from the nonreducing terminals of HA chains by hydrolysis activity. Simultaneously with this hydrolysis reaction, this enzyme could successively transfer the released disaccharides to the nonreducing terminal of the acceptor HA. The testicular hyaluronidase also acted on the GalNAcβ1-4GlcUA structure in chondroitin (Ch), chondroitin 4-sulfate (Ch4S), chondroitin 6-sulfate (Ch6S), and other GAGs as well as HA. Therefore, by repeating the transglycosylation using suitable combinations of Ch, Ch4S, Ch6S, and other GAGs as acceptors and donors, it was possible to custom synthesize GAGs. It is likely that application of this system would facilitate artificial reconstruction of GAG moieties of PG. We focused on decorin, which is a small and simple PG that has only one DS chain linked to its core protein, and used this method to reconstruct its GAG chains of decorin by first digesting them using the hydrolysis of testicular hyaluronidase. The obtained decorin as an acceptor and HA as a donor were incubated with testicular hyaluronidase under the condition of transglycosylation reaction. Judging from the analysis of the GAG chains in the transglycosylation reaction product, it was confirmed that an HA chain as a donor was transferred to the retained GAG chain of decorin as an acceptor. Similarly, it was possible to reconstruct the GAG chains in decorin to Ch, Ch4S, or Ch6S. Therefore, we succeeded in synthesizing an artificial family of decorin.

(542) **Array-Based Quantitative, Automated Analysis of Protein Glycosylation**

Johanna Griffin, Ruth Maya, Rakefet Rosenfeld, Zeev Smilansky, Yehudit Amor, Chani Rothmann-Scherz, Yossi Cohen, Mirit Kolog Gulko, Ana Moraga Grosz, Haim Bangio, Revital Rosenberg, Leonid Schvartzer and Ofer Markman
Procognia Ltd., Unit 4, The Switchback, Gardner Road, Maidenhead, Berkshire, SL6 7RJ, England.

Glycoproteins produced by mammalian cells are a mixture of glycoforms of the same protein with differing patterns of glycans. Most factors that influence cell growth likewise influence the glycoform mixture produced by the cells. Because glycosylation is not a template-guided process like transcription and translation and because glycans are diverse branched structures, the analysis of glycoforms remains one of the greatest challenges to the probability of success of biologic drugs. Procognia has developed lectin array and algorithm based technology to structurally analyze the glycans on intact, unpurified glycoproteins. The assay is rapid; up to 20 samples can be analyzed in parallel in approximately 2 hours. The analysis can be performed directly in culture medium or formulation without sample purification and without pretreatment or with only mild pretreatment. The results are accurate to within ±5%, but differences between samples of the same protein of <5% can be observed. The assay is easy to perform and no special training or analytic skills are required. The system is based on lectins arrayed in groups with overlapping recognition specificities. Glycoforms in a mixture bound to the lectin array are detected by labeled probes. Probes can be antibodies directed against the protein core or lectins that recognize glycans on the same glycoprotein, other than those bound to the arrayed lectins. A histogram of the normalized intensity for each lectin

or ‘fingerprint’ is characteristic of the glycan profile of the glycoform mixture and is highly sensitive to small differences in this profile among samples of the same protien. A proprietary knowledge base of lectin-glycan recognition behavior and proprietary algorithms are used to deconvolute fingerprint data to produce a quantitative analysis of glycan structures present in a glycoform mixture. This technology complements existing technology by providing substantially more data in ‘real time’ so that more informed decisions can be made during biologics discovery, development and manufacture. It is highly sensitive to glycosylation comparability among clones, cell lines, processes and batches of proteins. U-c fingerprint technology will likely increase the speed of development, the quality of the product and the probability of success of biologic pharmaceuticals.