ABSTRACTS SUBMITTED FOR THE 8TH ANNUAL CONFERENCE OF THE SOCIETY FOR GLYCOBIOLOGY

December 3–6, 2003
San Diego, California

ANNUAL CONFERENCE OF THE SOCIETY FOR GLYCOBIOLOGY

DECEMBER 3–6, 2003

CATAMARAN RESORT HOTEL, SAN DIEGO, CA

IMPORTANT DEADLINES

August 1
Submission of nominations for the Karl Meyer Award

August 29
Submission of abstracts

September 12
Submission of applications for the student travel stipends

October 31
Reservations at the Catamaran Resort Hotel

October 31
Advance conference registration

GENERAL CONFERENCE INFORMATION

CONFERENCE LOCATION

All technical sessions, posters, and exhibits will be at the Catamaran Resort Hotel, 3999 Mission Boulevard, San Diego, CA 92109. The Catamaran Resort Hotel offers casual elegance in a tropical paradise with acres of lush foliage and sun-drenched beaches. Mission Bay, the world's largest aquatic park, is at your doorstep and the enticing waves of the Pacific Ocean are just 100 yards away. The Catamaran Tower commands a view from the shores of La Jolla to Old Mexico and over Mission Bay to Point Loma and the spectacular San Diego skyline. Sea World, the world-famous San Diego Zoo and San Diego International Airport are just minutes away.

ACCOMMODATIONS

Hotel rooms for conference participants have been set-aside at the Catamaran Resort Hotel. To make a reservation, call the hotel directly, 858-488-1081 or (800) 422-8386. Be sure to mention that you are attending the Conference on Glycobiology in order to obtain the conference rate. A major credit card is required to guarantee a reservation. **The deadline for reservations at the conference rate is October 31.** Please make your reservations early, as the room block will sell out. The conference rates are: **$149 for single or double.** All rooms are subject to state and local taxes (currently 10.5%).

TRANSPORTATION

The Catamaran is located eleven miles from San Diego International Airport. Transportation is available via Cloud 9 Shuttle ($8.50 one way) and taxi (approximately $18-$24 one way).

PARKING

Valet parking is available for $10 per day. Self-parking is available for $8 per day for hotel guests.

PROGRAM CHAIR

Jeffrey D. Esko, University of California, San Diego
9500 Gilman Drive, La Jolla, CA 92093-0687
Tel: (858) 822-1100, president@glycobiology.org

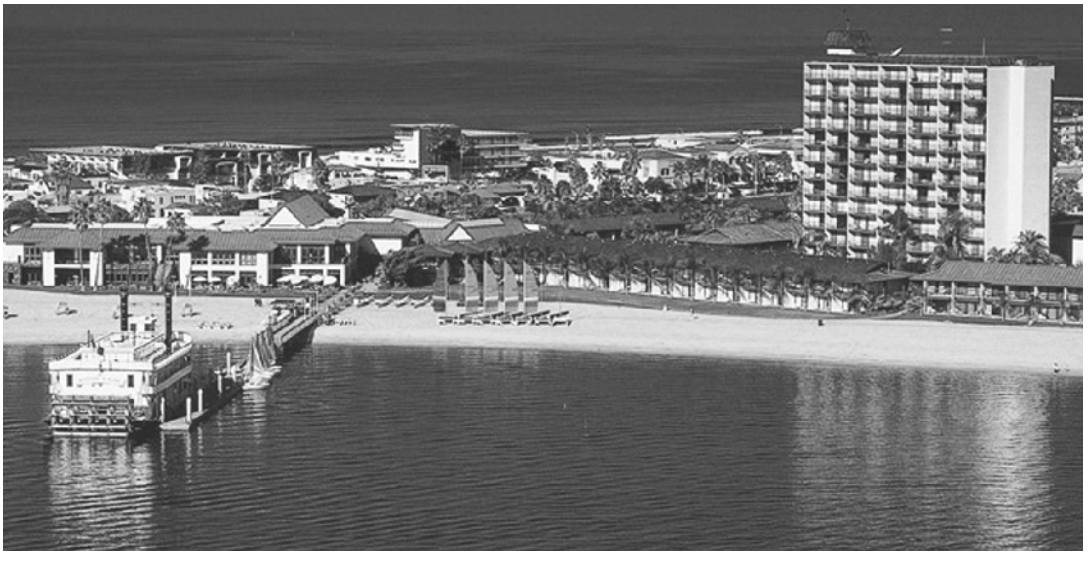
CONFERENCE CONTACT INFORMATION

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

HOTEL

The Catamaran Resort Hotel
3999 Mission Boulevard, San Diego, CA 92109
Tel: (858) 488-1081, (800) 422-8386
www.catamaranresort.com

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



PROGRAM OVERVIEW

Wednesday, Dec 3	9:00 am – 5:00 pm	**SATELLITE SYMPOSIUM I** **Human Disorders of Glycosylation.** Organized by Hudson H. Freeze, *The Burnham Institute* and Jeffrey Esko, *University of California, San Diego*
	9:00 am – 5:00 pm	**SATELLITE SYMPOSIUM II** **Production and Quality Control for Recombinant Glycoproteins in Biotechnological Uses.** Organized by Richard Cummings, *University of Oklahoma* and James C. Paulson, *Scripps Research Institute*
	7:00 – 7:15 pm	**CONFERENCE OPENING** Opening Remarks; **Jeffrey Esko,** *President, Society for Glycobiology*
	7:15 – 8:45 pm	**SESSION I: CARBOHYDRATE DIVERSITY** Chair: Gerald W. Hart, *Johns Hopkins School of Medicine*
	8:45 – 10:00 pm	**RECEPTION**
Thursday, Dec 4	8:30 – 10:00 am	**SESSION II: GLYCOBIOLOGY AND INFECTION** Chair, Marilynn Etzler, *University of California, Davis*
	10:00 – 10:30 am	Break and Exhibits
	10:30 am – 12:30 pm	**SESSION III: GLYCOBIOLOGY AND CELL BIOLOGY** Chair, Ajit Varki, *University of California, San Diego*
	12:30 – 2:00 pm	Lunch Break
	2:00 – 4:00 pm	**POSTERS and EXHIBITS. Poster Topics:** Carbohydrate Diversity, Glycobiology and Cell Biology, Glycobiology and Infection
	3:00 – 4:00 pm	**WORKSHOP: Informatics.** Anne Dell, *Imperial College*
	4:00 – 5:30 pm	**SESSION IV: GLYCANS IN PHYSIOLOGIC PROCESSES** Chair, Jamey Marth, *HHMI, University of California, San Diego*
Friday, Dec 5	8:30 – 10:00 am	**SESSION V: GLYCOBIOLOGY AND BIOCHEMISTRY** Chair: Chi-Huey Wong, *The Scripps Research Institute*
	10:00 – 10:30 am	Break and Exhibits
	10:30 am – 12:30 pm	**SESSION VI: GLYCOBIOLOGY AND IMMUNITY** Chair, Minoru Fukuda, *The Burnham Institute*
	12:30 – 2:00 pm	Lunch Break
	2:00 – 4:00 pm	**POSTERS and EXHIBITS Poster Topics:** Carbohydrate Diversity, Glycobiology and Cell Biology, Glycobiology and Infection, Glycans In Physiologic Processes, Glycobiology and Biochemistry, Glycobiology and Immunity
	3:00 – 4:00 pm	**DISCUSSION:** Reinvention of Study Sections and Your Grants Jeffrey Esko and Gerald W. Hart
	4:00 – 4:45 pm	**BUSINESS MEETING**
	4:45 – 5:30 pm	**KARL MEYER LECTURE**
	7:00 – 9:30 pm	**BANQUET.** Included with registration. Extra tickets for guests may be purchased.
Saturday, Dec 6	8:30 – 10:00 am	**SESSION VII: CHEMICAL GLYCOBIOLOGY** Chair, Richard Cummings, *University of Oklahoma Health Sciences Center*
	10:00 – 10:30 am	Break and Exhibits
	10:30 am – 12:30 pm	**SESSION VIII: GLYCOBIOLOGY AND DEVELOPMENT** Chair, Yu Yamaguchi, *The Burnham Institute*
	12:30 – 2:00 pm	Lunch Break
	2:00 – 4:00 pm	**POSTERS and EXHIBITS Poster Topics:** Carbohydrate Diversity, Glycobiology and Cell Biology, Glycobiology and Infection, Glycans In Physiologic Processes, Glycobiology and Biochemistry, Glycobiology and Immunity, Chemical Glycobiology, Glycobiology and Development, Glycobiology and Plasticity of Nervous and Musculoskeletal Systems
	4:00 – 5:30 pm	**SESSION IX: GLYCOBIOLOGY AND PLASTICITY OF NERVOUS AND MUSCULOSKELETAL SYSTEMS** *Chair, Hudson Freeze, The Burnham Institute*

CONFERENCE ON GLYCOBIOLOGY ADVANCE REGISTRATION FORM DEADLINE: October 31

ON SITE REGISTRATION. After October 31, please register on site. On site registration fees will be $50 more than advance registration. On site registration begins at 3 pm, Saturday, December 3 at the Catamaran Resort Hotel.
CANCELLATIONS. Notice of cancellation must be postmarked on or before November 28, 2003. A $40 processing fee will be deducted from refunds.

Name *Company/Institution*

Address *City, State, Zip/Postal Code/Country*

Phone *Fax* *Email*

SATELLITE SYMPOSIA (9 am - 5 pm, Saturday, Wednesday, December 3, $50)
Check one
☐ Human Disorders of Glycosylation
☐ Production and Quality Control for Recombinant Glycoproteins in Biotechnological Uses
Enter $50 for optional Satellite Symposium $ _____

CONFERENCE REGISTRATION **Enter registration amount checked below $ _____**
☐ $380, Member of the Society for Glycobiology ☐ $480, Non-Member
☐ $175, Student (Must be full-time in a Ph.D. program or within first two years of postdoctoral training)

EXTRA BANQUET TICKETS for GUESTS, 7 pm, Wednesday, December 3. 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 VISA or MasterCard. Sorry, no other cards are accepted.

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SPONSORS AND EXHIBITORS

The Society for Glycobiology gratefully acknowledges the support of the following companies:

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Wednesday, December 3, 2003
7:15 – 8:45 PM
CARBOHYDRATE DIVERSITY
Gerald W. Hart, Chair

Time *Abstract Number*

7:15 PM **Microarray Analysis of Glycosyltransferase Gene Expression**; James C. Paulson[1], Margarida Amado[1], Elena Comelli[1], Tim Gilmartin[2], Steven Head[2], Thomas Whisenant[2], Qi Yan[1], Mark Sutton[3] and Anne Dell[3]; *[1] Department of Molecular Biology and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, [2] DNA Core Facility, The Scripps Research Institute, La Jolla, CA, [3] Department of Biological Sciences, Imperial College, London, UK* 1

7:40 PM **Mass Spectrometry-Based Methods for Revealing the Glycans' Family Secrets;** Catherine E Costello[1,2], Mark E McComb[2], Peter B O'Connor[1,2] and Joseph Zaia[1]; *[1] Mass Spectrometry Resource, [2] Cardiovascular Proteomics Center, Boston University School of Medicine, Boston, MA 02118-2526* 2

8:05 PM **Glycosylation Techniques for Generating Carbohydrate Diversity**; Chi-Huey Wong; *Department Of Chemistry, The Scripps Research Institute, La Jolla, CA 92037* 3

8:30 PM **Heparanomics: A Voyage to Establish the Foundation of Glycobiology?**; Balagurunathan Kuberan, David Beeler, Miroslaw Lech, Zhengliang Wu, Roger Lawrence and Robert Rosenberg; *Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139* 4

8:35 PM **Application of Ion Trap MS^n Strategies to Structure Elucidation of Diverse Glycosylinositols Derived from Fungal Glycosphingolipids**; Suddham Singh, Vernon N. Reinhold, Beau Bennion and Steven B. Levery; *Department of Chemistry and Center for Structural Biology, University of New Hampshire, Durham, NH 03824-3598* 5

8:40 PM **High Throughput Glycan Array Analysis of Human Galectins-1 and –4.;** Richard A. Alvarez[1], Angela Lee[1], Ola Blixt[2], Padmaja Mehta[3] and Richard D. Cummings[1]; *[1] Protein-Carbohydrate Interaction Core H, Consortium for Functional Glycomics, and Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, [2] Carbohydrate Synthesis/Protein Expression Core D, Consortium for Functional Glycomics, and Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA, [3] Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK* 6

Thursday, December 4, 2003
8:30 – 10:00AM
GLYCOBIOLOGY AND INFECTION
Marilynn Etzler, Chair

Time *Abstract Number*

8:30 AM **Capsule Synthesis in the Pathogenic Fungus *Cryptococcus neoformans***; Amy J. Reese[1], Ulf Sommer[2], Hong Liu[1], Tricia R. Cottrell[1] and Tamara L. Doering[1]; *[1] Campus Box 8230, Washington University Medical School, 660 S. Euclid Ave., St. Louis, MO 63110, [2] Mass Spec Resource, Boston University School of Medicine, 715 Albany St., R-806, Boston, MA 02118* 7

8:55 AM **Regulation of Tissue Injury and Inflammation by Syndecan-1 Ectodomain Shedding**; Pyong Woo Park[1], William C. Parks[2], David B. Corry[1], Farrah Kheradmand[1] and Sheila Duncan[1]; *[1] Department of Medicine, Baylor College of Medicine, Houston, TX, [2] Department of Pediatrics, Washington University School of Medicine, St. Louis, MO* 8

9:20 AM **Reconstitution of GDP-Man Transport Activity with Purified *Leishmania* LPG2 Protein in Proteoliposomes**; Hiroaki Segawa[1], Stephen M. Beverley[2] and Salvatore J. Turco[1]; *[1] Dept. Biochemistry, University of Kentucky Med. Ctr., Lexington, KY 40536, [2] Dept. Mol. Microbiology, Washington Univ. Sch. Med., St. Louis, MO 63110*..... 9

9:45 AM **Cell Fusion and Syncytia Formation Is Mediated by Oligosaccharide Determinants of Nipah Virus Envelope F and G Glycoproteins and Can be Blocked by Lectins**; Ernie Levroney, Hector Aguilar, Kevin Gurney, Hector Valenzuela, Linda G. Baum and Benhur Lee; *Dept. of Microbiology, Immunology and Molecular Genetics and Dept. of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles CA*..... 10

9:50 AM **LacdiNAc Glycans Constitute a Parasite Pattern for Galectin-3-Mediated Immune Recognition.**; Timo K. van den Berg[1], Henk Honing[1], Niels Franke[1], Alexandra van Remoortere[2], Wietske E.C.M. Schiphorst[1], Cornelis H. Hokke[2] and Irma van Die[1]; *[1] Dept of Molecular Cell Biology & Immunology, VU University Medical Center, Postbus 7057, 1007 MB Amsterdam, the Netherlands, [2] Dept. of Parasitology, LUMC, Leiden, the Netherlands*..... 11

9:55 AM **Glycoproteins from Soluble Egg Antigens are the Major Antigens for Stimulation of Anti-glycan Antibody Responses in Primary *Schistosoma mansoni* Infections.;** A. Kwame Nyame and Richard D. Cummings; *Department of Biochemistry and Molecular Biology and Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK*..... 12

Thursday, December 4, 2003
10:30 AM– 12:30 PM
GLYCOBIOLOGY AND CELL BIOLOGY
Ajit Varki, Chair

Time *Abstract Number*

10:30 AM **The Nucleotide Sugar/Antiport System: What New Concepts Can We Learn from C. elegans?**; Carlos B Hirschberg.; *Department of Molecular and Cell Biology, Boston University Goldman School of Dental Medicine, Boston MA 02118*..... 13

10:55 AM **Molecular and Cellular Biology of Glycoprotein Quality Control;** Ying Wu[1], Matthew T. Swulius[1], Kelley W. Moremen[2] and Richard N. Sifers[1]; *[1] Depts of Pathology and Molecular & Cellular Biology, Baylor College of Medicine, Houston, TX, [2] Complex Carbohydrate Research Center, University of Georgia, Athens, GA* 14

11:20 AM **The Calnexin Cycle in Productive Glycoprotein Folding and Quality Control**; John J. Bergeron; *McGill University* 15

11:45 AM **The GGA Family of Coat Proteins Play a Critical Role in the Man-6-P Targeting Pathway**; Pradipta Ghosh[1], Balraj Doray[1], Janice Griffith[2], Hans J. Geuze[2] and Stuart Kornfeld[1]; *[1] Department of Medicine, Washington University School of Medicine, St.Louis,Mo.63110, [2] Department of Cell Biology,Utrecht University, Utrecht,Netherlands* 16

12:10 PM **Nuclear Localization of A Cytosolic Deglycosylating Enzyme Peptide:N-Glycanase (Pngase): Exploration of Possible Functions of Pngase in the Nucleus**; Samiksha Katiyar and William J. Lennarz; *Department of Biochemistry and Cell Biology and Institute of Cell and Developmental Biology, State University of New York at Stony Brook, Stony Brook, NY, 11794*..... 17

12:15 PM **O-GlcNAc: A New Paradigm for Modulating Cellular Responses to Stress.**; Natasha E Zachara, Chutikarn Butkinaree and Gerald W Hart; *Dept. Biological Chemistry, Johns Hopkins University School of Medicine, 725N. Wolfe Street, Baltimore, MD 21205-2185*..... 18

12:20 PM **Cog1 and Cog2 Deficiencies Prevent Normal Nucleotide Sugar Transport and Decrease Multiple Glycosyltransferase Activities;** Hudson H. Freeze, Ognian Bohorov, Xiaohua Wu and Erik Eklund; *The Burnham Institute, La Jolla CA*..... 19

Thursday, December 4, 2003
4:00 – 6:00 PM
GLYCANS IN PHYSIOLOGIC PROCESSES
Jamey Marth, Chair

Time *Abstract Number*

4 PM **Perception of the Glycan Signals that Initiate the Rhizobium-Legume Symbiosis.**; Marilynn E. Etzler, Biao Wu, Gurpreet Kalsi, Elaine Hoye and Bryan T. Harada; *Section of Molecular & Cellular Biology, University of California, Davis, CA 95616*..... 20

4:25 PM **Hyaluronan and CD44 Regulation of Tissue Injury and Repair**; Paul Noble.; *Yale University Pulmonary and Critical Care Section 441 TAC 333 Cedar St New Haven, CT 06520-8057*..... 21

4:50 PM **Physiologic functions of gangliosides**; Richard L. Proia.; *10 Center Dr MSC 1821, NIH, Bethesda, MD 20892*..... 22

5:15 PM **Fucosylation in the Control of Leukocyte Biology**; John B. Lowe; *HHMI, University of Michigan*..... 23

5:40 PM **Biologic Contribution of ST6Gal I Sialyltransferase in the Early Inflammatory Response**; Joseph TY Lau[1], Mehrab Nasirikenari[1], Julie R Ostberg[2] and Michelle M Appenheimer[1]; *[1] Dept. of Molecular and Cellular Biology, Roswell Park Cancer Institute, [2] Dept of Immunology, Roswell Park Cancer Institute* 24

5:45 PM **Selectin-Mucin Interactions: a Probable Molecular Explanation for the Association of Trousseau's Syndrome with Mucinous Adenocarcinomas**; Mark G Wahrenbrock, Lubor Borsig, Dzung Le, Nissi Varki and Ajit Varki; *Glycobiology Research and Training Center, University of California, San Diego* 25

5:50 PM **Modeling CDG Type I in *Caenorhabditis elegans*: A Screen for Modifying and Glycosylation-Dependent Loci.**; Kristin M.D. Shaw[1], William C. Wiswall Jr[1], Justin M. Prien[1], Justin M. Crocker[1] and Charles E. Warren[1,2]; *[1] Department of Biochemistry and Molecular Biology, [2] Genetics Program, University of New Hampshire* 26

Friday, December 5, 2003
8:30 – 10:00 AM
GLYCOBIOLOGY AND BIOCHEMISTRY
Chi-Huey Wong, Chair

Time *Abstract Number*

8:30 AM **Structural Basis of Glycolipid Presentation by CD1**; Dirk M. Zajonc[1], M.D. Max Crispin[1,3], Pauline M. Rudd[3], Raymond A. Dwek[3], Luc Teyton[2] and Ian A. Wilson[1]; *[1] Department of Molecular Biology and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 No. Torrey Pines Road, La Jolla, CA 92037, [2] Department of Immunology, The Scripps Research Institute, 10550 No. Torrey Pines Road, la Jolla, CA 92037, [3] Department of Biochemistry, University of Oxford, The Oxford Glycobiology Institute, South Parks Road, Oxford, OX1 3QU, UK*.... 27

8:55 AM **Structure and Function of Glycosyltransferases**; Masahiko Negishi.; *Pharmacogenetics section, LRDT, NIEHS, NIH, Research Triangle Park, NC 27709*..... 28

9:20 AM **New Mechanisms for the Enzymatic Cleavage of Oligosaccharides**; Stephen G Withers.; *Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, B.C., Canada V6T 1Z1*..... 29

9:45 AM **Sialic Acid Derivatives on Mucins of the Ocular Surface**; Pablo Argüeso and Ilene K. Gipson; *Schepens Eye Research Institute and Dept. of Ophthalmology, Harvard Medical School, Boston, MA*..... 30

9:50 AM **Characterisation of WlbA (a putative 3-dehydrogenase) and WlbC (a putative 3-transaminase) Two of the Key Enzymes Involved in *Bordtella pertussis* UDP D-Man-diNAcA Biosynthesis.**; Corin Wing[1], Velupillai Sri Kannathasana [1], Duncan Maskell[2], Andrew Preston[2], Cory Q Wenzel[3], Joseph S Lam[3] and Robert A Field[1]; *[1] Centre for Carbohydrate Chemistry, University of East Anglia, Norwich, Norfolk, NR4 7TJ, UK., [2] The Veterinary School, University of Cambridge, Cambridge, CB3 0ES, UK., [3] Department of Microbiology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada*..... 31

9:55 AM ***In vivo* Cleavage and Secretion of α2,6-Sialyltransferase**; Shinobu Kitazume[1], Yuriko Tachida[1], Ritsuko Oka[1], Eiji Miyoshi[2], Takaomi C. Saido[1] and Yasuhiro Hashimoto[1]; *[1] RIKEN Institute, [2] OsakaUniversity* 32

Friday, December 5, 2003
10:30 AM –12:30 PM
GLYCOBIOLOGY AND IMMUNITY
Minoru Fukuda, Chair

Time *Abstract Number*

10:30 AM **Regulation of Cell Surface Recognition Events Through Sulfation: Putting It On and Taking It Off**; Steven D. Rosen; *University of California, San Francisco*..... 33

10:55 AM **CD33-Related Siglecs in the Innate Immune system**; Paul Crocker; *Dundee University*..... 34

11:20 AM **Expression of GPI-Anchored Proteins: Events Post-Attachment of the Anchor En Route to the**

Cell Surface; Taroh Kinoshita, Satoshi Tanaka, Hisashi Ashida, Yuko Tashima and Yusuke Maeda; *Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*..... 35

11:45 AM **Selectin-Dependent Leukocyte Interactions with Vascular Surfaces**; Rodger P. McEver.; *Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, OK 73104* 36

12:10 PM **Siglec-7 and Siglec-9 Negatively Regulate T Cell Receptor Activation;** Yuzuru IKEHARA[1,2], Sanae K. IKEHARA[1,2] and James C. PAULSON[1]; *[1] Department of Molecular biology, MEM L71, The Scripps Research Institue, 10550 N. Torrey Pines Road, La Jolla, CA 92037, , [2] Present address; Div. Oncological Pathology, Aichi Cancer Center Research Institute, Aichi 464-0082, Japan* 37

12:15 PM **The Role of Extended Core 1 *O*-Glycans in Leukocyte Trafficking: b1,3-*N*-Acetylglucosaminyltransferase-3 is Essential for Synthesis of MECA-79 Antigen in Lymph Nodes and Plays a Role in Lymphocyte Adhesion to High Endothelial Venules**; Junya Mitoma, Hiroto Kawashima and Minoru Fukuda; *Glycobiology Program, Cancer Research Center, The Burnham Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037*..... 38

12:20 PM **Potent Suppression of Natural Killer Cell Response Mediated by the Ovarian Tumor Marker CA125;** Gary F. Clark[1], Jamie C. Morrison[1], Frank A. Lattanzio Jr.[1], Jing Yu[2], Yuping Deng[2], Nyet K. Wong[3], Howard R. Morris[3], Anne Dell[3] and Manish S. Patankar[1]; *[1] Department of Physiological Sciences, Eastern Virginia Medical School, Norfolk, VA, [2] The Glennan Center for Geriatrics and Gerontology, Department of Internal Medicine, Eastern Virginia Medical School, Norfolk, VA, [3] Department of Biological Sciences, Imperial College London SW7 2AZ, UK*..... 39

Saturday, December 6, 2003
8:30 – 10:00 AM
CHEMICAL GLYCOBIOLOGY
Richard Cummings, Chair

Time *Abstract Number*

8:30 AM **Frontal Affinity Chromatography/Mass Spectrometry in the Screening of Mixtures: Towards Determining the Binding Constant of an Undetectable Compound of Unknown Concentration**; Ole Hindsgaul.[1,2]; *[1] Carlsberg Laboratory, Copenhagen, Denmark, [2] The Burnham Institute, La Jolla, CA* 40

8:55 AM **Lessons From Nature: Antibiotic Optimization via Glycorandomization**; Jon S. Thorson.; *Laboratory for Biosynthetic Chemistry, University of Wisconsin School of Pharmacy, 777 Highland Avenue, Madison, WI 53705* 41

9:20 AM **Chemical Approaches To Studying Protein Glycosylation**; Carolyn R. Bertozzi.; *Departments of Chemistry and Molecular and Cell Biology and Howard Hughes Medical Institute, University of California, Berkeley, CA 94720-1460* 42

9:45 AM **A Novel Technology for Detection and Proteomic Analysis of O-GlcNAc Modified Proteins;** Animesh Nandi[1], Robert Sprung[1], Deb Barma[1], Yingxin Zhao[1], Mark A. Lehrman[2], John R. Falck[1] and Yingming Zhao[1,2]; *[1] Department of Biochemistry, UT Southwestern Medical Center, Dallas, TX 75390, [2] Department of Pharmacology, UT Southwestern Medical Center, Dallas, TX 75390*..... 43

9:50 AM **Glycosyltransferase Catalyzed Synthesis of Thiooligosaccharides**; Jamie R. Rich, Adam Szpacenko, Monica M. Palcic and David R. Bundle; *Alberta Ingenuity Center for Carbohydrate Science, Department of Chemistry, University of Alberta, Edmonton, AB, Canada* 44

9:55 AM **Chemoenzymatic Synthesis of Glycosaminoglycans with *Pasteurella* Synthases**; Paul L. DeAngelis[1], Wei Jing[2], Breca S. Tracy[1], Leonard C. Oatman[1] and Daniel F. Gay[1]; *[1] Dept. of Biochemistry and Molecular Biology, Oklahoma Center for Medical Glycobiology, Univ. of Oklahoma Health Sciences Center, 940 Stanton L. Young Blvd., Oklahoma City, OK 73104 , [2] Hyalose LLC, 655 Research Parkway, Oklahoma City, OK, 73104* 45

Saturday, December 6, 2003
10:30 AM – 12:30 PM
GLYCOBIOLOGY AND DEVELOPMENT
Yu Yamaguchi, Chair

Time *Abstract Number*

10:30 AM **Syndecans Control Matrix Deposition, Substrate-Dependent Cell Migration and Cell Non-Autonomous Left-Right Signaling**; H. Joseph Yost.; *Huntsman Cancer Institute, Center For Children, University of Utah, 2000 Circle of Hope, Salt Lake City, UT 84112 (www.hci.utah.edu/groups/yost/)*..... 46

10:55 AM **Egg Carbohydrates Inducing the Acrosome Reaction in Sea Urchin Sperm**; Victor D. Vacquier and Noritaka Hirohashi; *Marine Biology, Scripps Institution of Oceanography, UCSD, La Jolla, CA 92093-0202*..... 47

11:20 AM **Bt Toxin v. Worms: It All Comes Down To Sugars**; Joel S. Griffitts[1], Brad D. Barrows[1], Stuart M. Haslam[2], Anne Dell[1] and Raffi V. Aroian[1]; *[1] Secion of Cell and Developmental Biology, Univ California, San Diego, La Jolla, CA 92093-0349, [2] Department of Biological Sciences, Imperial College London, SW7 2AZ, London, UK*..... 48

11:45 AM **Regulation of Notch signaling by O-fucose Glycans**; Kenneth Irvine, Tetsuya Okajima, Nicola Haines, Liang Lei and Aiguo Xu; *Waksman Institute, 190 Frelinghuysen Rd, Piscataway NJ 08854*..... 49

12:10 PM **Chondroitin Is Required For *C. elegans* vulval Development and Embryogenesis**; Sara K. Olson[1], Ho-Yon Hwang[2], H. Robert Horvitz[2] 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] Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139*..... 50

12:15 PM **Trans-Cellular Induction of Neural-Specific Glycosylation by a Toll-Like Receptor**; Antti Seppo[1], Mary Sharrow[1], Parul Matani[1] and Michael Tiemeyer[2]; *[1] Yale University School of Medicine,*

[2] Complex Carbohydrate Research Center, University of Georgia 51

12:20 PM **Roles of O-Fucose Glycans in Ligand Binding to Mammalian Notch Receptors**; Kazuhide Uemura, Shaolin Shi and Pamela Stanley; *Department of Cell Biology, Albert Einstein College Medicine, New York, NY 10461* 52

Saturday, December 6, 2003
4:00 – 6:00 PM
GLYCOBIOLOGY AND PLASTICITY OF NERVOUS AND MUSCULOSKELETAL SYSTEMS
Hudson Freeze, Chair

Time *Abstract Number*

4 PM **Glycobiology of Neuronal Regeneration in the Adult CNS**; Alka A. Vyas[1], Ola Blixt[2], James C. Paulson[2] and Ronald L. Schnaar[1]; *[1] The Johns Hopkins School of Medicine, Departments of Pharmacology and Neuroscience, Baltimore, MD 21205, , [2] Scripps Research Institute, Departments of Molecular Biology and Molecular and Experimental Medicine, La Jolla, CA 92037* 53

4:25 PM **Polysialic Acid and Neural Plasticity**; Urs Rutishauser.; *Memorial Sloan-Kettering Cancer Center, New York, NY 10021* 54

4:50 PM **Broad Yet Surprisingly Specific Functions of Heparan Sulfate in Mammalian Brain Development**; Yu Yamaguchi; *The Burnham Institute* 55

5:15 PM **Glycosylation and Dystroglycan Function in Muscular Dystrophy**; Kevin Campbell; *University of Iowa College of Medicine* 56

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POSTER SESSION 2

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(1) **Microarray Analysis of Glycosyltransferase Gene Expression**

James C. Paulson[1], Margarida Amado[1], Elena Comelli[1], Tim Gilmartin[2], Steven Head[2], Thomas Whisenant[2], Qi Yan[1], Mark Sutton[3] and Anne Dell[3]
[1] Department of Molecular Biology and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, [2] DNA Core Facility, The Scripps Research Institute, La Jolla, CA, [3] Department of Biological Sciences, Imperial College, London, UK.

The availability of a highly annotated glyco-gene array produced by the Consortium for Functional Glycomics is beginning to yield a wealth of data concerning expression of glycosyltansferases in murine and human tissues and cell populations;
(http://web.mit.edu/glycomics/consortium/resources/resourcecoree.shtml).
This focused array has allowed evaluation of glycosyltransferase gene expression across all major families of enzymes, allowing investigations into the degree to which their expression is differentially regulated and, in conjunction with glycan profiling, how changes in gene expression are reflected in the glycan structures produced by cells. There are some major limitations to the analysis since some probe sets do not 'work' leaving gaps of information, and changes in the levels of gene expression alone are not necessarily predictive of changes in glycan synthesis. Nonetheless, information obtained from analysis of expression of glycosyltransferases in ten mouse tissues and in resting and activated murine lymphocytes illustrate the utility of this resource, and its potential for global analysis of glycosylation when coupled with glycan profiling of the same tissues and cells. [Supported by NIH Grants GM62116 (to the Consortium for Functional Glycomics) and GM60938 (to JCP), and a grant from the BBSRC (to AD)]

(2) **Mass Spectrometry-Based Methods for Revealing the Glycans' Family Secrets**

Catherine E Costello[1,2], Mark E McComb[2], Peter B O'Connor[1,2] and Joseph Zaia[1]
[1] Mass Spectrometry Resource, [2] Cardiovascular Proteomics Center, Boston University School of Medicine, Boston, MA 02118-2526 USA.

Alongside the spectacular recent growth in the use of mass spectrometry (MS) for the study of peptides and proteins that has laid the foundation for proteomics and for mapping of protein interaction pathways, significant progress has also been made in developing new approaches to structural and functional studies of oligosaccharides and glycoconjugates. These classes of molecules present even greater challenges to the analyst than do the linear biopolymers (proteins and oligonucleotides), because of the frequent heterogeneity of the glycan moieties, as well as the regular occurrence of branching and labile substituents. Bonds that cleave easily during sample preparation or analysis can rob the investigator of details that are critical for differentiating among multiple closely-related structures and thereby unraveling biologically important structure-activity relationships. Since these complex molecules carry information that governs intermolecular and intercellular interactions, transmit messages, contribute both to growth and development and to disease and degeneration, and enable the invasion of unfriendly organisms - yet also provide our means for defense, there is increasing appreciation of the need to determine their full structural details, despite the difficulties involved. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) enable powerful new mass spectral approaches to these studies. Using these ionization methods, we are establishing gentle conditions for the analyses of glycans that minimize sample decomposition and maximize sensitivity and the information content of the spectra. Areas of investigation include microscale sample manipulation and derivatization, the development of mild ESI conditions that minimize sulfate loss from glycosaminoglycans, the design of a system that allows direct MALDI examination of samples dispersed on thin layer chromatographic plates and other irregular surfaces, construction of a high pressure external MALDI source for the Fourier transform MS to stabilize fragile ions, and multiple approaches to tandem MS. In this lecture, some of the crucial steps in the development of current strategies for characterizing diversity among glycoproteins, glycolipids and oligosaccharides will be presented, with examples of their utilization chosen from ongoing applications to biological studies.

The BUSM MS laboratory is supported by grants from the National Institutes of Health, National Center for Research Resources and National Heart, Lung and Blood Institute.

(3) **Glycosylation Techniques for Generating Carbohydrate Diversity**

Chi-Huey Wong
Department Of Chemistry, The Scripps Research Institute, La Jolla, CA 92037

This lecture will present the current status of sugar array development and application and describe most recent advances in the field, especially focused on some unpublished work carried out in our laboratory.

(4) **Heparanomics: A Voyage to Establish the Foundation of Glycobiology?**

Balagurunathan Kuberan, David Beeler, Miroslaw Lech, Zhengliang Wu, Roger Lawrence and Robert Rosenberg
Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139 .

The ability to rapidly and easily synthesize the fragments of proteins and DNA has facilitated the establishment of structure-function relationship of these molecules and has also enabled genome-wide scans to be carried out. In the case of HS and other carbohydrates, total chemical synthesis is very challenging and has limited our knowledge of how these molecules orchestrate many different biologic systems. We have developed a general enzymatic approach for the rapid synthesis of bioactive HS. This approach should allow us to understand HS biosynthesis and to delineate the rules for HS-protein interactions. We initiated our explorations by carrying out the synthesis of ATIII-binding sites at both polysaccharide and oligosaccharide levels. This approach has been currently used in designing a diverse HS based oligosaccharide library that should be key to defining all interacting proteins and elucidating the chemical rules by which such interactions take place. Thus, our approach should establish a firm foundation for the glycobiology of proteoglycans.

(5) **Application of Ion Trap MS^n Strategies to Structure Elucidation of Diverse Glycosylinositols Derived from Fungal Glycosphingolipids**

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Fungi are important as bioreactors capable of carrying out useful chemical transformations on an industrial scale, as biotechnological platforms for heterologous expression of useful proteins and secondary metabolites, and as natural agents of bioremediation. They are also potential sources of novel enzymes and effector molecules. Useful products may encompass both glycosyltransferases and the glycoconjugates these are used to produce, including glycosphingolipids (GSLs). In addition, appreciation of fungi as agricultural, veterinary, and human pathogens has stimulated interest in fungal GSLs as potential targets for antifungal drugs, immunodiagnostic reagents, and synthetic vaccines. Studies of fungal GSLs have uncovered expression of two structural sub-classes, clearly divergent in key elements of their biosynthetic pathways, in their physico-chemical properties, and most likely in their functional roles. These sub-classes are: (i) monohexosylceramides, such as glucosylceramide (GlcCer) and galactosylceramide (GalCer); and (ii) glycosylinositol phosphorylceramides (GIPCs). While the former are similar to their mammalian counterparts, differing only in characteristic elements of ceramide structure, and in being accumulated by many fungi as biosynthetic end products, the latter have no mammalian counterparts, and are highly diverse with respect to glycan structure. Knowledge of possible functional roles for GIPCs is rudimentary, while the handful of studies carried out to date suggest a structural diversity potentially rivaling that already observed with mammalian GSLs, the breadth of which has only begun to be mapped. As with other complex glycoconjugates, detailed knowledge of structure is necessary for understanding GIPC biosynthesis and function, providing an essential compliment to genetic, proteomic, and immunological studies. NMR spectroscopy offers considerable potential for solving carbohydrate structural problems, in some cases enabling essentially complete derivation of glycan structure without resorting to other techniques. However, limitations on sample size frequently reduce the amount of information that can be acquired in practice. Methylation analysis is a traditional technique that provides unambigous glycosyl linkage information at high sensitivity, but offers no information regarding anomeric configuration, nor any indication of glycan sequence except for identification of non-reducing terminal residues. Mass spectrometry has long been used to derive information about glycan sequence at high sensitivity, and in recent years multistep disassembly of permethylated glycans by MS^n has shown considerable potential for deriving information about linkage and branching. However, capabilities of MS for identifying particular monosacharide residues and anomeric configurations have been slower to develop. More recently, MS^n studies comparing a variety of di-and trisaccharides of known structure indicate that clear and reproducible

differences in fragmentation may be observed solely as a function of differences in anomeric configuration as well as in linkage position. Development of an MS^n library will aid in the wider application of these techniques, and this library is being expanded to include glycosylinositols of increasing complexity derived from fungal GIPCs by high-temperature ammonolysis. This technique has been applied to glycosylinositols of GIPCs isolated from a variety of fungal sources, including *Aspergillus nidulans* and *Polyporus squamosus*.

(6) **High Throughput Glycan Array Analysis of Human Galectins-1 and –4.**

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Defining the specificity and affinity of glycan binding proteins (GBPs) to putative ligands is important to understanding cellular communication. This effort has been hampered by the difficulty in obtaining sufficiently diverse glycan structures to test current knowledge about ligand specificity. Recognizing this need, the National Institute of General Medical Sciences has funded an effort by the Consortium for Functional Glycomics to develop a high throughput, unbiased, global screening platform for the analysis of protein-carbohydrate interactions, which we call a glycan array. Populating the glycan array is accomplished by synthesis of glycans using chemical and enzymatic methods, as well as isolation of N- and O-glycans from glycoproteins, cells, and tissues. The glycans, with reducing sugars intact, are derivatized with an extended spacer arm containing a terminal biotin. Biotinylated glycans are arrayed on a 384 well streptavidin coated microplate at equal molar concentration and FITC-labeled GBPs are applied to measure binding specificity in relative fluorescence units. The array contains a growing diversity of glycans (150 to date), including mono-, di-, tri-, tetra-, hepta-saccharides, and further extended glycans, with and without fucose; O-glycopeptides; biantennary N-glycans; sulfated glycans; and multivalent polyacrylamide glycans. To test the utility of the glycan array we analyzed the binding specificity of human Galectin-1 (hGal-1) and human Galectin-4 (hGal-4). HGal-1 is a proto-type galectin with a single carbohydrate recognition domain (CRD) and is widely expressed as a dimeric protein (subunit ~14.6kDa). HGal-4 is a tandem repeat type galectin (35.5kDa), expressed primarily in the gastrointestinal tract, that contains one polypeptide and two CRDs. The differential localization of these proteins suggest alternative functions that may be reflected in their glycan specificity. Primary screening on the glycan array showed markedly different profiles of glycan binding for both galectins. HGal-4 bound to GlcNAcb3Galb4Glc- and O-glycopeptides with terminal galactose, whereas hGal-1 did not. Both bound glycans containing terminal galactose as expected. These findings were confirmed using a subset of glycans analyzed by surface plasmon resonance (SPR) on a Biacore 3000. These results demonstrate that the glycan array is a novel primary screening tool for identifying differences in GBP binding properties and will be important in expanding our understanding of GBP ligands and their biological functions.

(7) **Capsule Synthesis in the Pathogenic Fungus *Cryptococcus neoformans***

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Cryptococcus neoformans is a pathogenic yeast responsible for life-threatening disease in immunocompromised individuals [1]. It is distinguished from other pathogenic fungi by an extensive polysaccharide capsule surrounding its cell wall, which is absolutely required for virulence. The capsule is regulated in size by its environment, and has multiple adverse effects on the host immune system. About 90% of the capsule mass is composed of a polymer termed glucuronoxylomannan, or GXM. We have focused on this material with the dual goals of understanding its biosynthesis and incorporation into the extracellular capsule structure, and identifying ways to inhibit these processes [2]. GXM has a linear backbone of mannose residues linked α-1,3, which are modified with xylose (β-1,2 and β-1,4 linked) and glucuronic acid (β-1,2), and are 6-O-acetylated in a final ratio of (Man:Xyl:GlcA:Ac of 3:1-4:1:2) [3]. To investigate GXM synthesis we are investigating nucleotide sugar metabolism and transport, glycan synthetic reactions, and the process of capsule assembly. This presentation will focus on several areas. The first is studies of an α-1,3 mannosyltransferase we have purified, cloned and expressed, which shows strong homology to other proteins implicated in capsule synthesis. Additionally, studies of capsule assembly will be presented. We have shown that new capsule material is incorporated into the existing structure at the interface between the capsule and the cell wall. We have also found that the key cell surface feature required for interaction with capsule is α-1,3-glucan in the cell wall. Inhibition of synthesis of this moiety by double stranded RNA interference (targeting a putative α glucan synthase) yields slow-growing cells that generate capsule components but are not able to bind them, and are therefore acapsular [4]. As acapsular strains of C. neoformans are uniformly avirulent, these results suggest that α glucan synthase is a potential target for antifungal chemotherapy. Current efforts include determining the coding sequence of this large gene (estimated to be ~7 kb), localizing the protein, and generating a gene disruption. The disrupted strain will be used to confirm interference results and for virulence studies in mice. [Supported by NIGMS 66303, NIAID 49173, and the Burroughs Wellcome Fund.]

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(8) **Regulation of Tissue Injury and Inflammation by Syndecan-1 Ectodomain Shedding**

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Cell surface heparan sulfate proteoglycans (HSPGs) have been implicated in the pathogenesis of various infectious and non-infectious inflammatory disorders. However, the molecular mechanisms of how HSPGs regulate the outcome of these processes have yet to be clearly defined. To address these issues, we examined the role of syndecan-1, the predominant cell surface HSPG of epithelia, in murine models of lung injury and inflammatory diseases. At the cell surface, syndecan-1 functions as a co-receptor for various ligands, such as matrix components, cytokines and growth factors, by catalyzing the formation of ligand-signaling receptor complexes. Syndecan-1 also functions as a soluble HSPG because it can be proteolytically cleaved from the cell surface by a mechanism known as ectodomain shedding. Our results show that syndecan-1 shedding is markedly activated in murine models of tissue injury and inflammation. In the bleomycin-induced acute lung injury model, shedding of syndecan-1 ectodomains by matrix metalloproteinase-7 (MMP-7) generates a chemokine gradient that guides the egression of neutrophils into the alveolar compartment. In this mechanism, bleomycin injury induces expression of MMP-7 and the CXC chemokine KC by alveolar epithelial cells. Newly synthesized MMP-7 and KC then bind to the heparan sulfate (HS) chains of syndecan-1, MMP-7 sheds the syndecan-1/KC complex, and the complex translocates into the alveolar space to generate a transepithelial chemokine gradient for neutrophil migration. Consistent with these results, MMP-7 null mice instilled with bleomycin do not shed syndecan-1 ectodomains. Furthermore, bleomycin-instilled syndecan-1 null and MMP-7 null mice show significantly reduced levels of KC and neutrophils in their bronchoalveolar lavage fluids relative to wild type control mice, indicating that lung epithelial syndecan-1, MMP-7, and KC coordinately function to regulate and confine inflammation to specific sites of tissue injury. Interestingly, syndecan-1 shedding also regulates CXC chemokine-mediated neutrophil migration in acute bacterial pneumonia. Airway levels of KC and neutrophils are significantly reduced in syndecan-1 null mice intranasally-instilled with *Pseudomonas aeruginosa* compared to wild type controls. In stark contrast, our results show that in the model of allergic lung disease, syndecan-1 shedding is activated in response to allergen instillation but it attenuates leukocyte recruitment to the lung. Furthermore, relative to wild type mice, allergen-challenged syndecan-1 null mice show markedly exacerbated allergic lung disease features, such as enhanced airway hyperresponsiveness, glycoprotein secretion, eosinophilia, and Th2 cell lung recruitment. These inflammatory indices are significantly suppressed when mice are co-instilled with allergen and purified syndecan-1 ectodomains or heparin, but not ectodomain core protein that are devoid of HS. Our results also demonstrate

that ectodomain HS chains bind and inhibit several Th2 chemokines (CCL7, CCL11, CCL17). These data suggest that one of the mechanisms how syndecan-1 shedding attenuates allergic lung inflammation is by inhibiting chemokine-mediated Th2 cell lung recruitment. Taken together, these findings indicate that syndecan-1 shedding coordinates with other inflammatory mediators to regulate lung inflammation by controlling leukocyte migration. Depending on the type of the initial insult and the chemokine that syndecan-1 ectodomains regulate, syndecan-1 shedding can be a pro- or anti-inflammatory mechanism.

(9) **Reconstitution of GDP-Man Transport Activity with Purified *Leishmania* LPG2 Protein in Proteoliposomes**

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The synthesis of glycoconjugates within the secretory pathway of eukaryotes requires the provision of lumenal nucleotide-sugar substrates. This is particularly important for pathogenic microbes, such as the protozoan parasite *Leishmania*, because of the synthesis of considerable quantities of glycoconjugates that play critical roles in its infectious cycle. We previously isolated a Leishmania gene called *LPG2* that encodes a ~37 kDa protein, containing up to nine transmembrane domains. The encoded protein was found to be a GDP-Man transporter, which functions as a multi-specific oligomer of LPG2 subunits. To purify the transporter, a poly histidine tagged LPG2 (LPG2cHis6) construct was transfected in a LPG2-/- strain of *Leishmania donovani* and the LPG2cHis6 complex was purified in a functional form by metal chelate columns. The purified LPG2cHis6 complex was reconstituted into artificial liposomes consisting of egg yolk phosphatidylcholine using polystyrene SM-2 beads. The proteoliposomes exhibited GDP-Man transport activity with an apparent Km value similar to transport activity in crude microsomes from wildtype cells. The transport activity was enhanced by GMP preloading with no divalent cation requirement and showed multi-substrate specificity. Importantly, reconstitution of transport activity using purified LPG2 protein in liposomes provides firm experimental evidence regarding its autonomous nature. Thus, these studies have important implications to the structure and function of nucleotide-sugar transporters not only in *Leishmania* but also in other eukaryotes.

(10) **Cell Fusion and Syncytia Formation Is Mediated By Oligosaccharide Determinants of Nipah Virus Envelope F and G Glycoproteins and Can Be Blocked by Lectins**

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The Nipah virus (NiV) causes fatal hemorrhagic encephalitis in 40% of infected patients, and is classified as a Category C priority pathogen in the NIAID Biodefense Research Agenda. Endothelial cell fusion and syncytia formation is a pathologic hallmark of Nipah viral disease, and membrane fusion is strictly mediated through the F and G envelope glycoproteins. Since glycosylation is known to affect the activity and function of viral envelope glycoproteins, we sought to identify oligosaccharide determinants that are critical for F and G glycoprotein expression and function. Lectin blots indicated that the F glycoprotein has abundant high mannose N-glycans, and some complex N-glycans that appear to be capped with α(2,6)-linked sialic acid. In contrast, the G glycoprotein has primarily complex N-glycans, as well as abundant asialo O-glycans. Altered electrophoretic mobility of the Nipah G protein produced in cells treated with an O-linked glycosylation inhibitor also indicated the presence of O-glycans on the G protein, consistent with the presence of a mucin-like serine/threonine rich stalk domain in G. Point mutations abolishing N-glycan attachment sites were made in the F and G proteins, that have 8 and 6 potential N-linked glycosylation sites, respectively. Loss of specific N-glycans allowed protein expression but abolished syncytia formation of 293T cells, demonstrating that certain N-linked sites play critical roles in the fusion process. Given the role of oligosaccharides in mediating cell fusion and syncytia formation, we asked whether specific lectins could block cell fusion. Galectin-1, a mammalian lectin that binds to galactose residues on N- and O-glycans, and is expressed by activated endothelial cells and dendritic cells, dramatically inhibited the efficiency of cell fusion mediated by F and G glycoproteins. Similarly, cyanovirin, a high mannose specific lectin with anti-viral activity against HIV, also had a potent inhibitory effect on cell fusion mediated by the Nipah virus F and G glycoproteins. Since fusion is a co-operative process mediated by both F and G glycoproteins, our inhibition results with galectin-1 and cyanovirin suggest that different glycans on the F and G glycoproteins play critical and complementary roles in Nipah virus fusion. In addition to the effects on syncytia formation, galectin-1 addition to dendritic cells resulted in a dramatic increase in the pro-inflammatory cytokines IL-6 and IL-1β. Dendritic cells cultured with galectin-1 demonstrated a 1000-fold increase in IL-6 secretion and a 40-fold increase in IL-1β secretion, compared to cells cultured in media alone. As IL-6 and IL-1β have been shown to be critical determinants of survival in patients infected with hemorrhagic fever viruses, these results indicate that galectin-1 may have a protective role in Nipah virus infection. Significantly, the ability of endogenous lectins like galectin-1 to inhibit Nipah virus fusion, coupled with the increasing number of lectins found on cognate cell types such as dendritic cells, suggest that the glycan-lectin interface on the cell surface is a critical component of our innate immune response against viral pathogens.

(11) **LacdiNAc Glycans Constitute A Parasite Pattern for Galectin-3-Mediated Immune Recognition.**

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The innate immune system is equiped with a variety of receptors and soluble molecules that recognize invading pathogens by pathogen-associated molecular patterns (PAMPs) such as toll-like receptors and lectins. Because parasitic helminths and their secretory products often exhibit foreign glycosylation patterns, as compared to their vertebrate hosts, their glycans constitute attractive candidates for recognition by the innate immune system. Recently we showed that the dendritic cell-associated C-type lectin DC-SIGN recognizes the glycan antigen Le^x on egg glycoproteins of schistosomes (1). Here, we focus on immune recognition of schistosome glycans by macrophages. While Galβ1-4GlcNAc (LacNAc, LN) is the most common constituent of N-linked glycans on vertebrate proteins, helminth glycans more often are based on GalNAcβ1-4GlcNAc (LacdiNAc, LDN) units. Since *Schistosoma mansoni* infected mice generate antibodies to LDN determinants (2), we postulated that LDN might be a molecular pattern for immune recognition. Using LDN-based affinity chromatography and mass spectrometry we have identified galectin-3 as the major LDN binding protein in macrophages. Surface plasmon resonance and ELISA analysis demonstrated that recombinant galectin-3 binds to neoglycoconjugates carrying LDN antigens. In a solid-phase adhesion assay galectin-3 binds *S. mansoni* egg antigens (SEAs) and a monoclonal antibody against the LDN glycan inhibits this binding, suggesting that LDN antigens within SEAs contribute to galectin-3 binding. By immunocytochemical analysis of *S. mansoni* infected mice high levels of galectin-3 were detected in liver granulomas, and co-localization of galectin-3 and LDN glycan epitopes was observed on parasite egg shells. In an in vitro assay, galectin-3 mediates recognition and phagocytosis of LDN-coated particles by macrophages. These findings provide evidence that LDN-glycans constitute a parasite pattern for galectin-3-mediated immune recognition and suggest that galectin-3 may function in the presentation of schistosome egg antigens to the immune system.

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(12) **Glycoproteins from Soluble Egg Antigens are the Major Antigens for Stimulation of Anti-glycan Antibody Responses in Primary *Schistosoma mansoni* Infections.**

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Schistosoma mansoni is one of three major metazoan parasites that cause the disease schistosomiasis. Schistosomes have a complex life cycle that alternates between vertebrates and specific freshwater snail intermediate hosts. The development of vertebrate stage parasites goes through a progression from cercariae, the infective larvae, to schistosomula, the undifferentiated juveniles, to adult male and females who produce fertilized eggs. Strong Th-2 type anti-

glycan humoral immune responses are generated during the course of infection in vertebrates. Among the targets of host anti-glycan responses are Lewis x (Le^x; Galβ1-4(Fucα1-3)GlcNAc-R), LacdiNAc (LDN; GalNAcβ1-4GlcNAc-R) and Fucosylated LacdiNAc (LDNF; GalNAcβ1-4(Fucα1-3)GlcNAc-R). We have used synthetic neoglycoconjugates and monoclonal antibodies to the glycans in ELISA to determine the kinetics of antibody responses during *S. mansoni* infection in mice and to study its relationship to the expression of the glycans on soluble glycoproteins from the different vertebrate stage parasites. We find that infected mice generate both IgM and IgG antibodies to all three glycans. However, other then a small IgM response to Le^x observed around week 4-5 post-infection, the peak antibody responses to all three glycans occur at wk 8-13, long after the parasites have developed to egg laying adults. Analysis of soluble saline extracts from cercariae, schistosomula, adults and eggs by ELISA using both IgM and IgG monoclonal to Le^x, LDN and LDNF shows that the three glycans are abundantly expressed on soluble extracts from eggs. In contrast, the glycans are undetectable in extracts from cercariae, schistosomula and adults, except for a small expression of Le^x in cercarial extracts. Thus, the pattern of expression of the three glycans on soluble material from the developing parasites directly correlates with the pattern of antibody responses observed during infection and strongly suggests that soluble egg glycoproteins are the major source of glycan antigens in primary schistosome infections. Consistent with this conclusion, we detect no anti-glycan antibodies in sera from mice infected with irradiated cercariae, which develop to non-egg-laying adults. These results provide an explanation for the survival of parasites during primary schistosome infections because the potential immune response against the parasites occur long after they have developed from immuno-susceptible larvae to immuno-resistant adults.

(13) **The Nucleotide Sugar/Antiport System: What New Concepts Can We Learn From C. elegans?**

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The Nucleotide Sugar Transport/Antiport System:What New Concepts Can We Learn From C. elegans? Carlos B. Hirschberg, Department of Molecular and cell Biology, Boston University Goldman School of Dental Medicine, Boston, MA 02118 Approximately forty percent of proteins in eukaryotes are membrane bound and secreted and undergo post-translational modifications in the lumen of endoplasmic reticulum and Golgi apparatus prior to reaching their final destination within or outside cells. Nucleotide sugars, nucleotide sulfate and ATP, substrates for the above reactions, must first be transported into the lumen of these organelles by transporters in a coupled exchange with the corresponding nucleoside monophosphate, an antiporter. Recently we have been studying novel roles of this transport/antiport cycle in the morphogenesis, behavior and stress response of Candida albicans and C. elegans.In C. albicans we found that deletion of the Golgi GDP/UDPase encoding gene GDA1, which is necessary for generating the nucleoside monophosphate antiporter, results in hypoglycosylation of O-linked glycoproteins and partial impairment in the yeast-hyphal transition. In the C.elegans genome we found three ORFs encoding proteins containing apyrase conserved regions homologous to the S. cerevisiae GDP/UDPase GDA1; one of these was cloned, expressed in yeast and found to have its active site facing a lumenal compartment and its transcript upregulated under conditions of stress. Finally, a mutant of C. elegans, with an altered surface lectin binding phenotype and resistant to bacterial infection was found to be impaired in a novel dual substrate nucleotide sugar transporter. Together these results and the occurrence of other nucleotide sugar transporters and putative antiporter generators in C. elegans demonstrate the usefulness of this metazoan to study roles of glycosylation in multicellular eukaryotes.

(14) **Molecular and Cellular Biology of Glycoprotein Quality Control**

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Because biosynthetic quality control machinery can dictate the fate of newly synthesized proteins that transit through the secretory pathway, it is now evident that the severity of a genetic disease is not entirely dependent on the inherited DNA sequence. Delineation of protein quality control is expected to identify novel avenues for therapeutic intervention of numerous loss-of-function and gain-of-toxic-function disorders that arise from effective intracellular proteolysis, or the lack thereof, respectively. Mutant forms of the secretory protein α1-antitrypsin are retained in the early secretory pathway and can become substrates for distinct proteolytic systems. Impaired secretion and intracellular degradation are risk factors for the development of pulmonary emphysema (loss-of-function) and liver cirrhosis (gain-of-toxic-function), respectively. Asparagine-linked glycosylation occurs during translocation into the endoplasmic reticulum and generates an appendage through which a distinct set of processing enzymes and lectins can mediate folding of the attached polypeptide or orchestrate its disposal if structural maturation is not achieved. Recent evidence indicates that modification of the oligosaccharides by endoplasmic reticulum I (ERManI), when bound to nonnative protein structure, completes the formation of a "disposal signal" which is then recognized by a cognate receptor. The rules and molecular logic that govern the accuracy of quality control, its participation in the eukaryote gene expression surveillance system, and capacity to modify certain genetic diseases will be discussed.

(15) **The Calnexin Cycle in Productive Glycoprotein Folding and Quality Control**

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No abstract available

(16) **The GGA Family of Coat Proteins Play a Critical Role in the Man-6-P Targeting Pathway**

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In higher eukaryotic cells, the sorting of newly synthesized acid hydrolases to lysosomes is dependent on the mannose 6-phosphate (Man-6-P) recognition system. A key step in this pathway is the binding of the Man-6-P-tagged hydrolases to Man-6-P receptors (MPRs) in the trans-Golgi network(TGN). The receptors are then packaged into transport vesicles for delivery to lysosomes. Earlier immune electron microscopy studies localized the MPRs to adaptor protein-1 (AP-1) clathrin-coated vesicles at the TGN, implicating this coat protein in transport vesicle assembly. Recently we and others have demonstrated that the MPRs also bind to the GGA family of multi-domain proteins in the TGN. This binding involves the interaction of the VHS domains of the GGAs with an acidic cluster-dileucine sorting motif on the cytoplasmic tails of the MPRs. Mutations in the AC-LL motif impair acid hydrolase sorting and decrease binding of the MPRs to the GGAs, but not to AP-1, indicating that the GGAs have an essential role in the sorting process. We have found that the GGAs and AP-1 co-localize in clathrin-coated buds at the TGN and that the hinge domains of the GGAs bind to the γ-appendage of AP-1. Further, AP-1 contains a bound form of casein kinase-2 that phosphorylates the GGA hinge domains,causing autoinhibition that could induce the release of the MPRs from the GGAs, resulting in the directed transfer of the MPRs from the GGAs to AP-1. Chemical cross-linking studies demonstrated that the three mammalian GGAs form a complex upon recruitment onto Golgi-enriched membranes while depletion of any one GGA by RNA interference resulted in mislocalization of the other GGAs from the TGN to the cytosol. This was associated with impaired incorporation of the MPRs into clathrin-coated vesicles at the TGN and missorting of cathepsin D. These findings indicate that the three mammalian GGAs cooperate with each other and with AP-1 to package MPRs into transport vesicles at the TGN.

(17) **Nuclear Localization of A Cytosolic Deglycosylating Enzyme Peptide:N-Glycanase (pngase): Exploration of Possible Functions of pngase in the Nucleus**

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Introduction:
Yeast peptide:*N*-glycanase (yPNGase) has been reported to be a cytosolic enzyme involved in the deglycosylation of misfolded glycoproteins in ERAD pathway (1). Yeast PNGase interacts with the 19S proteasome subunit via Rad23 (nuclear excision repair protein) (2). However the mouse homolog (mPNGase) of yPNGase interacts not only with Rad23 homolog (mHR23B) but also with several other proteins including mS4 (19S proteasome subunit), AMFR (Autocrine Motility Factor Receptor), Y33K (a hypothetical protein), Ub (Ubiquitin) and Importin α -2 (Nuclear Pore Complex protein) using two hybrid screen(3). Physical interaction of mPNGase with importin α -2, mS4 and mHR23B has been confirmed using GST pull down assays. Interaction of mPNGase with importin raised the possibility of its nuclear localization and

involvement in other processes. Immunofluorescence experiments using HeLa cells showed the presence of mPNGase in the nucleus. Currently the investigation of its co-localization and co-immunoprecipitation with mS4 is being carried out to examine the possibility of its involvement in proteasome dependent degradation pathway of small (<45 kda) misfolded glycoproteins which might shuttle between the nucleus and cytosol in an energy independent manner (4). Nuclear co-localization and co-immunoprecipitation of mPNGase with mHR23B (which has already been reported to localize in the nucleus (5)) is also underway to explore the role of mPNGase in the nucleus. The other possibility for its presence in the nucleus would be its involvement in cell cycle regulation. We plan to carry out cell cycle synchronization to identify possible additional unknown functions of mPNGase. (Supported by GM33184 to WJL)

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(18) **O-GlcNAc: A New Paradigm for Modulating Cellular Responses to Stress.**

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Stress, whether environmental, physiological, or chemical, induces multiple signal transduction events which modulates the response of cells/organisms to deleterious conditions. We have recently shown that in response to multiple forms of stress, cells rapidly and dynamically increase global levels of nucleocytoplasmic glycosylation, O-GlcNAc. In addition, in response to many forms of stress levels of the O-GlcNAc transferase increase. Increasing O-GlcNAc levels by over expression of the O-GlcNAc transferase or inhibition of O-GlcNAcase increases a cells tolerance to lethal stress treatments. While deletion of OGT results in cells which are more sensitive to stress. O-GlcNAc mediated stress tolerance appears linked to the ability of cells to produce heat shock proteins; in cells with increased O-GlcNAc heat shock proteins are induced faster in response to stress, while in cells where O-GlcNAc has been reduced the levels of heat shock proteins are reduced. Moreover, in cells treated with lethal doses of stress, O-GlcNAc levels first increase and then rapidly decrease suggesting that O-GlcNAc is a pro-survival mechanism and that removal of O-GlcNAc is a key step in the apoptotic cascade. We report here our current studies aimed at understanding how stress modulates the levels and activities of OGT/O-GlcNAcase; and how O-GlcNAc modulates the expression of heat shock proteins. (Supported by NIH CA42486). G.W.H. has the potential to receive royalties from Covance Research Products and Hoffmann - La Roche for sales of the CTD 110.6 antibody.

(19) **Cog1 and Cog2 Deficiencies Prevent Normal Nucleotide Sugar Transport And Decrease Multiple Glycosyltransferase Activities**

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Previous studies showed that CHO cell mutants *ldlB* and *ldlC* make incomplete N-and O-linked oligosaccharides lacking terminal galactose and/or sialic acid residues. The ldlB and ldlC gene products belong to a Golgi-associated, cytosolic 8-subunit Conserved Oligomeric Golgi (COG) complex that may be involved in Golgi assembly and/or intracellular trafficking. We studied both Cog1-deficient (ldlB) and Cog2-deficient (ldlC) lines to understand how these glycosylation defects arise, and focused on nucleotide sugar transporters (NST) and glycosyltransferases involved in O-glycan biosynthesis. We developed a new method to measure NST activity using Streptolysin-O permeabilized cells and membrane-permeant GalNAc-alpha-phenyl acceptor. By this method, CMP-NeuAc transporter activity had a Km of 2.0uM, was destroyed by TritonX-100, and was absent in CMP-NeuAc transporter-deficient CHO Lec2. UDP-Gal transporter activity had a Km of 1.0 uM, was sensitive to TritonX-100 and was absent in UDP-Gal transporter-deficient CHO Lec8 cells. Cog1- and Cog2- deficient cells had substantially reduced transporter activities. Both were almost undetectable in Cog1. CMP-NeuAc transporter activity was decreased 6-fold in Cog2-deficient cells. Cog1 and Cog2 deficiency also reduced the activities of ST3Gal-I and Core1GalT. Cog1 deficient cells had only 25% ST3Gal-I and 16% Core1GalT activities, while Cog2 deficient cells had 65% and 83% of these activities, respectively. Complementation with the corresponding cDNAs normalized ST3Gal-I and NST activities.

ER to Golgi trafficking of at least one glycosyltransferase was impaired in Cog1 and Cog2 mutants. Cog1 deficient cells over-expressing ST3Gal-I tagged with GFP accumulated the protein mainly within ER, while CHO cells localized it to Golgi. In Cog2-deficient cells, the tagged protein resided in both ER and Golgi. We suggest that Cog1 and Cog2 defects result in retention of ST3Gal-I-GFP in ER rather than allowing its trafficking to or accumulation in Golgi.

The synthesis of galactosaminoglycans, was also studied by metabolic labeling with 35SO4 and 3H-glucosamine, as well. Cog1-deficient cells showed reduced incorporation into galactosaminoglycans (17% 35S and 37% 3H compared to control) indicating that both sulfation and chain elongation were affected.

Together these results indicate that even though Cog1 and Cog2 reside in the same nominal molecular complex, they have different effects on glycosylation that include reducing NST activities, donor substrate availability, localization of glycosyltransferase to Golgi and the steady state activities of multiple glycosyltransferases. Our data support previous studies showing that Cog1 and Cog2 deficiencies affect other peripheral and integral Golgi-associated proteins. Loss of either of these COG subunits leads to an unstable COG complex that no longer localizes on the cytosolic face of the Golgi. This, in turn, affects the intracellular distribution of multiple components of the glycosylation biosynthetic machinery. (Supported by RO1 DK55615)

(20) **Perception of the Glycan Signals That Initiate the Rhizobium-Legume Symbiosis.**

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Rhizobia secrete lipochitooligosaccharide signals, called Nod factors, that initiate the plant responses that lead to the establishment of the rhizobium-legume symbiosis. Previous studies from our laboratory have identified a Nod factor-binding protein, called LNP1, that also has nucleotide phosphohydrolase activity. LNP1 is a peripheral membrane protein present on the surface of young root hairs and undergoes a redistribution to the tips of the root hairs in the presence of symbiotic rhizobia or carbohydrate ligand. This protein appears to function in Nod factor perception as part of a receptor complex, and transformation of a nonleguminous plant with LNP alone confers on the plant the ability to undergo root hair swelling and deformation in specific response to carbohydrate ligand or rhizobial symbionts of the legume from which the LNP was obtained. Results from ongoing studies on native and mutant forms of LNP1 will be discussed in assessing the mechanism by which this protein operates. (This work was supported by NIH Grant GM21882)

(21) **Hyaluronan and CD44 Regulation of Tissue Injury and Repair**

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A hallmark of tissue and injury and repair is the turnover of extracellular matrix components. Following non-infectious lung injury there is an accumlation of lower molecular mass hyaluronan (HA) that is subsequently cleared prior to completion of the repair process. We have investigated the roles of HA and CD44 in regulating lung injury and repair following fibrotic injury. CD44 null mice were found to succumb to unremitting inflammation following intratracheal administration of bleomycin. Coincident with the inflammation was a failure to clear HA from the lung tissue. Generation of chimeric mice that had bone marrow repletion of CD44 null mice with wild type CD44 bone marrow rescued the inflammatory phenotype. Targeted overexpression of HAS2 to the lung under the direction of a lung-specific promoter protected mice from the severity of the initial insult of lung injury but resulted in increased fibrosis at later time points. Finally, recognition that HA signaling was preserved in CD44 null macrophages led us to investigate the role of the fundamental innate immunity receptor family, the Toll-like receptors (TLRs) in mediating HA signaling. We have identified a novel requirement for both TLR2 and TLR4 in mediating HA signaling in vitro and lung injury and repair in vivo. Together, these studies illustrate the important role for HA and HA receptors in regulating tissue injury and repair. This work has been supported by NIH grants HL-57486 and AI-52478.

(22) **Physiologic Functions of Gangliosides**

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Gangliosides -- a subfamily of the larger group of glycosphingolipids -- are composed of a ceramide anchor attached to an oligosaccharide chain of variable complexity. They are distinguished by the presence of one or more sialic acid residues. The abundance and diversity of gangliosides is greatest in the nervous system. However, these lipids are found on all mammalian cell plasma membranes where they appear to be concentrated within microdomains specialized for cell signaling. Gangliosides have been implicated as playing roles in fundamental cell processes such as growth, differentiation and adhesion. We have generated a series of knockout mice with gene disruptions in the ganglioside biosynthetic pathway. Individually, and when combined, these mutations result in mice that lack subsets of gangliosides allowing investigations into their roles in physiology. These mice display a broad array of phenotypes that affect developmental processes, metabolism, and central nervous system function and stability. The results point to potential roles of ganglioside in diseases affecting the central nervous system, in cancer and in diabetes.

(23) **Fucosylation in the Control of Leukocyte Biology**

John B. Lowe
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No abstract available

(24) **Biologic Contribution of ST6Gal I sialyltransferase in the Early Inflammatory Response**

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The synthesis of the common and well-documented Siaa2,6 to Galb1,4GlcNAc structure (Sia6LacNAc), is principally mediated by the sialyltransferase ST6Gal I. While the requirement for ST6Gal I during B cell activation is already well recognized, the widespread expression of ST6Gal I outside of the B cell compartment and the intricate manner in which its expression is regulated strongly suggest additional roles for this sialyltransferase. In liver, elevation of hepatic and serum ST6Gal is part of the acute phase reaction, the hepatic response to inflammatory stimuli, and is governed by the inducible, liver-specific promoter-regulatory region, P1. We have generated a mutant mouse, Siat1dP1, with a specific disruption to the P1 region. These animals display reduced ST6Gal I mRNA in liver and concomitantly reduced sialyltransferase activities in liver and serum. Siat1DP1 mice exhibit profoundly more severe peritonitis from i.p. injection of Salmonella typhimurium. During thioglycollate-elicited peritonitis, 7-fold greater rate of leukocyte accumulation in the peritoneum is consistently observed in the first 5 hours of thioglycollate injection when compared to identically treated wild-type cohorts. However, the mechanistic basis of how abrogation of P1 can lead to the observed aberrantly robust leukocyte response is unclear, and our observations may suggest recruitment of P1 for ST6Gal I expression in processes critical to inflammatory cell function.

(25) **Selectin-Mucin Interactions: A Probable Molecular Explanation for the Association of Trousseau's Syndrome with Mucinous Adenocarcinomas**

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In 1865, Dr. Armand Trousseau described spontaneous, recurrent superficial migratory thrombophlebitis associated with occult cancers, and this was later correlated with disseminated microangiopathy (platelet-rich clots in small blood vessels). Trousseau's syndrome often occurs in association with mucinous adenocarcinomas, which secrete abnormally glycosylated mucins and mucin fragments into the bloodstream. Since carcinoma mucins can have binding sites for selectins, we hypothesized that selectin-mucin interactions might trigger this syndrome. Early reports and some medical textbooks suggest that carcinoma mucins can initiate coagulation by direct activation of clotting factor X. However, contamination by tissue factor (TF) and/or other bioactive molecules likely confounded such studies. This is not surprising, since intact mucins are large complex molecules that can associate non-covalently with many tissue-derived lipids and proteins.

Proteolytic fragments of mucins are often released into the blood by tumor cells, and the heavily glycosylated domains of mucins are resistant to proteases, because of shielding by the clustered O-glycans. Taking advantage of the protease resistance, we developed a novel method for the purification of large mucin fragments from xenografted tumors - defined as >200 kDa domains sufficiently glycosylated as to be protected from the action of a broad-spectrum protease. The rationale for the overall approach was to eliminate all contaminating lipids and polypeptides, including procoagulant molecules such as phospholipids, platelet activating factor and TF. Also, the final product would be a mixture of proteolytic fragments of various mucin polypeptides, similar to that secreted by naturally occurring cancers. The complete procedure involved extensive lipid extraction and broad-spectrum protease digestions followed by other enzyme treatments to remove large polyanionic molecules such as glycosaminoglycans and nucleic acids, and eventual gel filtration to collect the surviving high molecular weight mucin fragments.

When such highly purified, well-characterized, tissue-factor free, carcinoma mucin preparations were intravenously injected into mice, platelet-rich microthrombi were rapidly generated. This pathology was markedly diminished in P- or L-selectin deficient mice. Heparin (an antithrombin-potentiating agent which can also block P- and L-selectin recognition of ligands) ameliorated this platelet aggregation, but had no additional effect in P- or L-selectin deficient mice. Inhibition of endogenous thrombin by co-injection of recombinant hirudin with mucin also did not block platelet aggregation. Mucins generated platelet aggregation in vitro in hirudinized whole blood, but not in platelet-rich leukocyte-free plasma, nor in whole blood from L-selectin deficient mice. Thus, Trousseau's syndrome is likely triggered by interactions of circulating carcinoma mucins with leukocyte L-selectin and platelet P-selectin, without requiring accompanying thrombin generation. The mechanism of L-selectin action remains obscure, but presumably involves some form of rapid signaling response to ligation by the mucin ligands. This data can also explain why heparin ameliorates Trousseau's syndrome, while Vitamin K antagonists that merely depress thrombin production do not.

(26) **Modeling CDG Type I in *Caenorhabditis elegans*: A Screen for Modifying and Glycosylation-Dependent Loci.**

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The type I congenital disorders of glycosylation (CDG-I) result from defects in the addition of *N*-glycans to nascent glycoproteins. All subtypes are severe multisystemic syndromes but clinical presentations are highly variable even within a subtype. The pleiotropic nature of the defects is consistent with the disruption of numerous glycoproteins that are critical to a wide variety of specific physiological processes. We postulate that the broad spectrum of clinical presentations is a consequence of the primary genetic defect interacting with other alleles in an individual's genetic background to exacerbate particular symptoms and mitigate others. The problem is two-fold: To identify the glycoproteins that control particular symptoms and to determine which secondary genetic loci interact to influence the pathology.

We observed that RNAi disruption of oligosaccharide transfer and that tunicamycin inhibition of lipid-linked oligosaccharide (LLO) biosynthesis is embryonic lethal in *C. elegans*. However, at marginally sub-lethal doses of tunicamycin, animals hatch but arrest as L1 (Lva). The effect is partially reversible; some hatchlings resume normal development when picked to drug-free media. At lower doses, the drug causes a slow-growth (Gro) phenotype, the expressivity of which is dose-dependent but otherwise the animals are grossly wild type. Thus, the response of *C. elegans* to tunicamycin replicates CDG-Ij. We reasoned that by screening for tunicamycin hypersensitive mutants, we would isolate alleles of 3 classes: Mutations affecting tunicamycin pharmacokinetics, non-epistatic components of the *N*-glycoprotein biosynthesis apparatus (glycosylation pathway, unfolded protein response etc) and genes encoding glycan-dependent proteins. By characterizing alleles from the latter 2 classes of synthetic phenotypes, we could determine which secondary genetic loci interact to influence the severity of the response to a systemic defect in glycosylation and identify the glycan-dependent genetic pathways that control particular phenotypes. Alleles in these classes would be distinguishable from those that alter tunicamycin metabolism using RNAi with a component of the LLO biosynthetic pathway.

Growing wildtype animals on NGM containing 2µg/ml tunicamycin at 20ºC slows the generation time by 1 day. We grew synchronized populations of EMS-treated F2 or F3 hatchlings on NGM containing 2µg/ml tunicamycin and picked those that were still L1 or L2 after 60 hours at 20ºC to drug-free plates. After another 48 hours at 20ºC, animals that resumed development to become L4 were cloned and lines established. From ~60,000 haploid genomes screened in the F2 generation, 8 genetically independent mutants were

recovered. From ~90,000 haploid genomes screened in the F3 generation, 24 genetically independent alleles have been isolated. Of these alleles: 4 F2 isolates were synthetic Lva and 4 were synthetic Gro with tunicamycin. Of the F3 alleles 12 were synLva and 12 were synGro. Four mutations that show synthetic phenotypes with defects in *N*-glycosylation were selected for initial characterizations, and mapping and cloning of these candidates is proceeding.

(27) **Structural Basis of Glycolipid Presentation by CD1**

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The human CD1 family of antigen presenting cell surface receptors consists of five members, CD1a to CD1e. These proteins bind a variety of different self and foreign lipid and glycolipid antigens and present them to CD1-restricted T cell receptors (TCRs) for specific recognition. All bound ligands observed so far bind with their lipid moieties buried deep within the CD1 binding groove, whereas the sugar headgroup is exposed at the protein surface for recognition by the TCR. Human CD1a and mouse CD1d show a relatively simple organization of their binding groove with two pockets A' and F', that each accommodate one alkyl chain of the lipid, whereas human CD1b has additional pockets that presumably account for the extra mass of longer alkyl chain lipids of up to 80 carbons. We have recently determined the crystal structure of CD1a in complex with a self-antigen glycosphingolipid sulfatide. The lipid adopts an S-shaped conformation with the sphingosine chain completely buried in the A' pocket and the fatty acid chain emerging from the interface of the A' pocket into the more exposed F' pocket. The headgroup is anchored in the A'-F' junction and protrudes into the F' pocket for TCR recognition. The CD1a structure suggests that its binding groove is specifically designed for preferentially binding and presenting lipid or glycolipid antigens of an intermediate length (34-42 carbon atoms total) to CD1a-restricted T cells. Because the A' pocket is quite narrow with a fixed terminus, it can act as a molecular ruler to select out alkyl chains of this particular length. The cellular co-localization of the CD1 antigens and the lipid ligands in combination with the structural differences of the different binding pockets throughout the CD1 family are the major factors that determine which types of glycolipids are bound by which CD1 protein. The three-dimensional structure of various CD1-glycolipid complexes are now shedding light on the structural differences that define the binding specificity for CD1 antigens.

(28) **Structure and Function of Glycosyltransferases**

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The X-ray crystal structures of β1,3-glucuronyltransferase 1 (GlcAT 1) and α1,4-*N*- acetylhexosaminyltransferase (EXTL2) have now been determined in the presence of both donor and acceptor substrates. These enzymes are involved in glucosaminylglycan synthesis where they catalyze inverting and retaining transfer reactions, respectively. As members of a large family of enzymes that transfer sugar groups from donor nucleotide-sugars to acceptor substrates, GlcAT1 and EXTL2 retain conserved GT folds with two subdomains. The N-terminal subdomain binds to the donor and the C-terminal one to the acceptor, while transfer reaction occurs a cleft formed between the two subdomains. The structures revealed residues that may be involved in transfer reaction and those that interact directly with substrates. Adaptive binding of the disaccharide moiety of the acceptor sugars enables the enzymes to catalyze either an inverting S_N2-type displacement reaction or a retaining S_Ni-like transfer reaction.

(29) **New Mechanisms for the Enzymatic Cleavage of Oligosaccharides**

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Mechanisms for the enzymatic hydrolysis of glycosides by glycosidases are now well-established and supported by substantial structural and mechanistic analyses. These involve acid/base-catalysed cleavage by a single-displacement mechanism or by a double-displacement mechanism via a glycosyl-enzyme intermediate. Another well-established, but minimally mechanistically characterized, mechanism of bond cleavage is that carried out by the lyases that cleave uronic acid-containing polysaccharides, forming a 4,5-unsaturated sugar. Two new mechanisms for bond cleavage have recently been uncovered and will be described. One of these is that of the α-glucan lyases, which cleave starch via a 1,2-elimination to produce a 2-hydroxyglucal product. The other is of the Family 4 glycosidases, which require a divalent metal ion and NAD, and which are shown to actually carry out an elimination/addition mechanism. Kinetic, structural and mechanistic evidence will be presented, along with some speculation on evolutionary origins.

(30) **Sialic Acid Derivatives on Mucins of the Ocular Surface**

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Sialic acids comprise a large family of derivatives of neuraminic acid containing methyl, acetyl, sulfate and phosphate among other groups, that confer specific physicochemical properties (i.e. hydrophilicity) to the molecules carrying them. Several years ago, we developed a monoclonal antibody, designated H185, distribution of which was altered in the ocular surface of patients with dry eye disease. Recent findings using immunoprecipitation and immunodepletion techniques have demonstrated that the H185 antigen is carried by the membrane-associated mucin MUC16 in human corneal epithelial cells. Both the H185 epitope and MUC16 mucin were found in human tears from normal eyes, as demonstrated by ELISA (H185) and Western blot analysis (H185, MUC16). Digestion of tears with neuramidase from A. ureafaciens (wide linkage-specificity) abolished H185 antibody binding to human tears, indicating that the H185 epitope is a sialic acid residue on MUC16. Digestion of tears with neuraminidases from C. perfringens and S. pneumoniae (restricted linkage-specificity) did not affect or minimally affected H185 binding to tears, suggesting that the sialic acid residue responsible for H185 binding may be substituted. As determined by HPAEC-PAD, a single peak with the characteristics of O-acetyl sialic acid was produced after treatment of tears with neuraminidase from A. ureafaciens, but not after treatment with C. perfringens. De-O-acetylation of tears resulted in a 65% decrease in H185 binding. These results indicate that the H185 antigen at the ocular surface is an O-acetylated sialic acid derivative and implies that O-acetyl sialic acids on mucins may have a role in maintaining a hydrated ocular surface. Supported by NIH/NEI Grant R01EY03306 to IKG.

(31) **Characterisation of WlbA (a putative 3-dehydrogenase) and WlbC (a putative 3-transaminase) Two of the Key Enzymes Involved in *Bordtella pertussis* UDP D-Man-diNAcA Biosynthesis.**

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Bordetella pertussis is responsible for the childhood disease whooping cough and, as is increasingly recognised, the cause of chronic cough in adults.[1] Current vaccines are generally highly effective, but on occasion cause side effects.[2] In addition, the re-emergence of whooping cough in vaccinated populations has been observed in countries, such as the Netherlands.[3] There is a strong case to be made that whooping cough vaccines would benefit from additional components that would increase efficacy. One candidate is the polysaccharide (PS) moiety of lipopolysaccharide (LPS), which is not present in any current vaccine.[4]

B. pertussis LPS consists of a lipid A domain attached to a branched-chain core oligosaccharide (band B LPS), that may be further substituted by a complex trisaccharide referred to as band A consisting of N-acetylglucosamine (GlcNAc), 2,3-dideoxy-2,3-di-N-acetylmannosaminuronic acid (Man-2,3-diNAcA) and 2-N-acetyl-4-N-methylfuco-disamine (Fuc2Nac4NMe). Some of the genes in the *wlb* gene locus (wlbA-wlbL),[1] which is involved in band A trisaccharide expression, have been cloned and over-expressed in *Escherichia coli*.[5] The original assignment for the *wlb* genes was based on sequence homology studies between the *wlb* genes in *B. pertussis* and related organisms.[1,5,6]

WlbA was originally proposed to catalyse the conversion UDP-D-Man-di-NAc to UDP-D-Man-di-NAc-A and WlbC was proposed to be a *trans*aminase, which generates an amino group at the 3 position of UDP-D-Man-NAc to give UDP 3-amino-3 deoxy-D-ManNAc. However, WlbA has no homology to dehydrogenases that generate carboxylic acids and WlbC could not *trans*aminate an alcohol to an amine as such reactions proceed from ketones. Therefore it is apparent that an oxidation step must be required. In a revised pathway we propose that WlbA oxidizes UDP-GlcNAc to UDP-3-keto-GlcNAc, prior to the *trans*aminase action of WlbC. Our current results reveal

that WlbA preferentially uses UDP-uronic acids as substrates in the presence of NAD^+ and WlbC preferentially uses L-glutamate as its amine donor.

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(32) ***In Vivo*** **Cleavage and Secretion of α2,6-Sialyltransferase**

Shinobu Kitazume[1], Yuriko Tachida[1], Ritsuko Oka[1], Eiji Miyoshi[2], Takaomi C. Saido[1] and Yasuhiro Hashimoto[1]

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The deposition of amyloid β-peptide (A-β) in the brain is a hallmark of Alzheimer's disease pathogenesis. A-β is generated from the amyloid precursor protein (APP) by the sequential actions of proteases, β-secretase (BACE1) and gamma-secretases. BACE1, a membrane-bound aspartic protease, is considered to be a major therapeutic target of Alzheimer's disease. We previously found that BACE1 also cleaved a membrane-bound sialyltransferase, ST6Gal I [1]. By BACE1 overexpression in COS cells, the secretion of ST6Gal I markedly increased and the amino terminus of the secreted ST6Gal I started at Glu-41 (E41-form). Here we report that E41-form of soluble ST6Gal I is present in rat plasma, suggesting that BACE-dependent cleavage occurs also in vivo [2]. In addition, we found another plasma isoform of soluble ST6Gal I, which is cleaved at different site from E41. Plasma ST6Gal I is mainly derived from the liver and its level is reported to increase during the process of inflammation. We have analyzed the plasma ST6Gal I isoforms in the experimental hepatitis, and found marked changes of the isoform ratio in addition to the high levels of the enzyme. Isoform analysis may have a diagnostic value for monitoring pathogenic processes of hepatic diseases. References: 1. S. Kitazume et al. (2001) Alzheimer's β-secretase, β-site amyloid precursor protein-cleaving enzyme, is responsible for cleavage secretion of a Golgi-resident sialyltransferase. Proc. Natl. Acad. Sci. U.S.A. 94, 3650-3655. 2. S. Kitazume et al. (2003) Characterization of α2,6-sialyltransferase cleavage by AlzheimerÕs β-secretase(BACE1) . J. Biol. Chem., 278, 14865-71.

(33) **Regulation of Cell Surface Recognition Events Through Sulfation: Putting It On and Taking It Off**

Steven D. Rosen

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No abstract available

(34) **CD33-Related Siglecs in the Innate Immune System**

Paul Crocker

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No abstract available

(35) **Expression of GPI-Anchored Proteins: Events Post-Attachment of the Anchor En Route to the Cell Surface**

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The attachment of glycosylphosphatidylinositol (GPI)-anchor is a post-translational modification that is widely used in eukaryotes. GPI glycolipids are synthesized and en bloc attached to proteins in the endoplasmic reticulum (ER). GPI-anchored proteins (GPI-APs) are subsequently subjected to post-attachment modification of the GPI and then transported into the membrane microdomains so-called rafts, where they are thought to play roles in signal transduction. Little is known how GPI-APs are modified and transported to rafts, and what genes are involved in these post-attachment regulations. To clarify these events, we have established mutant CHO cell lines that are defective in the post-attachment pathway. Here, we report characteristics of two mutant cell groups and genes responsible.

First mutant, C10 cells express GPI-APs at the wild-type level but those GPI-APs are totally resistant to PI-specific phospholipase C (PI-PLC), which releases most of the GPI-APs from the wild-type CHO cells. This mutant cell is defective in the removal of an acyl chain from the inositol of GPI that normally occurs in the ER immediately after attachment of the anchor to proteins. We cloned a responsible gene termed *PGAP1* (for Post GPI Attachment to Proteins 1). *PGAP1* encodes an ER-resident membrane protein that has a domain conserved in various lipases, suggesting that PGAP1 is the inositol deacylase. In this mutant, transport of GPI-APs from the ER to Golgi was much slower than in wild-type cells. Therefore, the removal of acyl group from the inositol ring is required for efficient transport of GPI-APs to Golgi.

Second mutant cells are defective in the surface GPI-APs expression but are normal in biosynthesis and attachment of GPI-anchors to proteins. GPI-APs in these mutant cells were transported at a normal rate from the ER to late Golgi/Trans Golgi Network and then rapidly disappeared from the cells due to the secretion into the culture medium. The secretion was associated with the cleavage of the GPI portion that occurred at a post-Golgi site. GPI-specific phospholipase D (GPI-PLD), which does not cleave GPI-APs on the wild-type cells unless detergent is added or rafts are impaired, enhanced the cleavage on the mutant cells. We cloned a responsible gene, termed *PGAP2* that encodes a Golgi-resident membrane protein. Since a defect in PGAP2 rendered GPI-APs susceptible to GPI-PLD, PGAP2 may be involved in the modification and/or incorporation into rafts of GPI-APs. Thus, Golgi protein PGAP2 regulates expression and location of GPI-APs on the cell surface.

(36) **Selectin-Dependent Leukocyte Interactions with Vascular Surfaces**

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Binding of selectins to cell-surface ligands mediates rolling of leukocytes on activated platelets and endothelial cells and on other leukocytes. Selectins also support rolling of platelets on activated endothelial cells and may help stabilize platelet aggregates. These multicellular adhesive interactions contribute to immune surveillance and to responses to tissue injury and infection. The leukocyte mucin PSGL-1 mediates interactions with L-selectin on other leukocytes and with P-selectin on activated platelets and endothelial cells. Both P- and L-selectin bind in a stereochemically precise manner to a small N-terminal region of PSGL-1 that must be modified with three tyrosine sulfates and a core-2 O-glycan capped with the sialyl Lewis x determinant. However, P- and L-selectin differ in the kinetics and affinity with which they bind to PSGL-1. Both molecular and cellular features affect selectin-dependent rolling under flow. One important cellular feature is the extrusion of long, thin membrane tethers, which reduce force on adhesive bonds and increase the probability of forming additional bonds along the tether. Tether extrusion from neutrophils rolling on P-selectin is dynamic, increasing rapidly as wall shear stress increases and decreasing rapidly as wall shear decreases. Tether formation correlates closely with slower, more stable leukocyte rolling. Neutrophils that roll longer also form more complex tethers, which may further stabilize rolling. Both P-selectin and PSGL-1 are dimers; this favors formation of dimeric bonds between these molecules. Associations of the cytoplasmic domains of P-selectin and PSGL-1 with cytosolic components may promote bond clusters between cell-surface patches of these proteins. The kinetic and mechanical properties of selectin-ligand interactions also modulate rolling. At low force regimes, increasing wall shear stress prolongs the lifetimes of bonds between PSGL-1 and either P-selectin or L-selectin; these counter-intuitive interactions are called catch bonds. Further increases in wall shear stress shorten lifetimes; these interactions are called slip bonds. A minimum shear threshold must be reached to support L-selectin-dependent rolling. This phenomenon requires a shear rate-dependent transport effect that allows new bonds to form before the original bonds dissociate and a shear stress-dependent effect that stabilizes L-selectin/PSGL-1 interactions by prolonging the lifetimes of catch bonds.

(37) **Siglec-7 and Siglec-9 Negatively Regulate T Cell Receptor Activation**

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The human siglec family of cell receptors is comprised of eleven members of the Ig superfamily which are functionally related by their ability to bind sialic acid containing carbohydrates of glycoproteins and glycolipids as ligands. The siglecs are predominantly expressed in immune cells, and contain consensus immune-globulin receptor family tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domains. To date, while CD22 (Siglec-2) is known to negatively mediate B cell receptor signaling through recruitment of SHP-1 via its ITIM motif, the regulatory functions of other siglecs is just beginning to be explored.

Siglec-7 and Siglec-9 are highly homologous ÔCD33Õ related siglecs and are expressed on NK cells. It is known that Siglec-7 can inhibit NK cell cytolysis activity, and growth of CD34 positive hematopoietic precursors and chronic myeloid leukemia cells following antibody cross-linking (J Exp Med 190:793-802, 1999; PNAS. 96:15091-15096. 1999; PNAS. 98:5764-5769. 2001). Both

Siglec-7 and SiglecÐ9 have also been detected on CD8+ T cells (J Biol Chem 274:34089-34095. 1999; J Biol Chem 275:22121-22126, 2000) and are partially co-localized with the T cell receptor (TCR) when stably expressed on Jurkat (2002 Glycobiology meeting). We therefore hypothesized that they might also participate in negative regulation of TCR activation.
Using Jurkat T cell leukemia cell lines stably transfected with FLAG tagged Siglec 7 or Ð9, we demonstrated that both Siglecs could recruit SHP-1 through the increased phosphorylation following either pervanadate stimulation or TCR engagement. Phosphorylation of Tyr319 on ZAP-70, which plays a pivotal role in up-regulation of IL2 transcription following TCR stimulation, was decreased in both Siglec-7 or Ð9 expressing Jurkat cells compared to mock transfected cells. This correlated with lower transcriptional activity of nuclear factor of activated T cells (NF-AT) as determined using a luciferase reporter gene. Alanine substitution of the conserved Arg in the sialic acid binding site of Siglec-7 (Arg^{124}) or Siglec-9 (Arg^{120}) was previously shown to abolish binding to sialic acid containing ligands of both receptors (Glycobiology 10:431-438, 2000; J Biol Chem. 275:22127-22135, 2000). Comparison of the effects of the corresponding mutants with native Siglec-7 and Siglec-9 in Jurkat cells revealed reduced inhibitory function in the NF-AT/luciferase transcription assay, suggesting that ligand binding is required for optimal inhibition of TCR signaling.
The results show that both Siglec-7 and -9 can negatively regulate TCR activation of Jurkat cells by recruitment of SHP-1, and that a functional ligand-binding domain is required for optimal activity. Using FACS analysis we confirmed that Siglec-7 and -9 were found on subsets of both ab and gd T cells in peripheral blood leukocytes from healthy donors, although the expression frequencies (5-18%) varied from donor to donor. The results suggest that these Siglecs may participate in modulating the activation threshold of T cells expressing them. (Supported by NIH grants GM-60938 and AI-050143, and by Uehara Memorial foundation Research Fellowship)

(38) **The Role of Extended Core 1 *O*-Glycans in Leukocyte Trafficking: b1,3-*N*-Acetylglucosaminyltransferase-3 Is Essential for Synthesis of MECA-79 Antigen in Lymph Nodes and Plays A Role in Lymphocyte Adhesion to High Endothelial Venules**

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The initial step of lymphocyte homing to peripheral lymph nodes (PLN) is mediated by the interaction between L-selectin on lymphocytes and peripheral node addressin (PNAd) on high endothelial venules (HEV). It has been well demonstrated that MECA-79 antibody inhibits not only *ex vivo* binding of lymphocytes to HEV of PLN, but also lymphocyte homing *in vivo* (1). We previously showed that 6-sulfo sialyl Lewis x in extended core 1 structure, NeuNAca2-3Galb1-4(Fuca1-3(Sulfo-6))GlcNAcb1-3Galb1-3GalNAca1-Thr/Ser, which is recognized by MECA-79 antibody, plays a role as L-selectin ligand in HEV(2). We also demonstrated that sialyl Lewis x in extended core 1 structure without 6-sulfation expressed on PSGL-1 in leukocytes can serve as L-selectin ligand (3). Thus the extended core 1 *O*-glycan appears to be an important core structure, in addition to core 2 branch, for L-selectin ligand in various cells. b1,3-*N*-acetylglucosaminyltransferase-3 (b3GlcNAcT-3), one of the seven b3GlcNAcTs, is required for synthesis of the extended core 1 structure, by adding a GlcNAc residue to core 1 *O*-glycan via b1,3-linkage (2, 3).
To gain further insights into synthesis and function of the extended core 1 *O*-glycans, including MECA-79 antigen, we generated mice deficient in b3GlcNAcT-3. We replaced b3GlcNAcT-3 gene with cDNA encoding enhanced green fluorescent protein (EGFP) to monitor the expression of b3GlcNAcT-3 *in vivo*. b3GlcNAcT-3 (-/-) mice developed normally and lacked any obvious anomaly. As expected, EGFP expression was observed in HEV. Leukocytes including T cells, B cells, granulocytes and natural killer cells, were also EGFP-positive, although the expression level was much less than HEV. Interestingly, MECA-79 reactivity was completely absent in PLN of b3GlcNAcT-3 (-/-) mice, demonstrating that b3GlcNAcT-3 is the only b3GlcNAcT which can add GlcNAc to core 1 *O*-glycan in b1,3-linkage. Moreover, the binding of L-selectin-IgM chimera to HEV was appreciably reduced, and Stamper-Woodruff assay revealed that lymphocytes failed to bind to PLN prepared from b3GlcNAcT-3 (-/-) mice *in vitro*. These results indicate that the formation of extended core 1 structure is essential for synthesis of functional L-selectin ligand in PLN. Further *in vivo* analysis such as lymphocyte homing assay is now being performed. Supported by NIH grant CA48737.
(1) Streeter et al, J. Cell Biol. **107,** 1853-1862, 1988
(2) Yeh et al., Cell **105,** 957-969, 2001
(3) Mitoma et al, J. Biol. Chem. **278**, 9953-9961, 2003

(39) **Potent Suppression of Natural Killer Cell Response Mediated by the Ovarian Tumor Marker CA125**

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CA125 is a useful diagnostic marker for epithelial ovarian cancer (EOC). We recently characterized the N- and O-linked oligosaccharides expressed on CA125 using ultrasensitive mass spectrometric methods (Wong, N. K. et al., (2003) *J. Biol. Chem.*, **278**, 28619-28634). The majority of the N-glycans associated with this mucin are bisecting type biantennary, triantennary, and tetraantennary glycans. These N-glycans have previously been associated with the suppression of NK cell cytotoxicity. We therefore investigated the effect of CA125 on NK cells using established *in vitro* assays. Here we report that CA125 is a very potent inhibitor of NK cell mediated cytotoxicity at physiologically relevant concentrations found in ascites and cyst fluids. This effect is not due to decreased proliferation or apoptosis of NK cells. Exposure of NK cells to CA125 leads to a 40-70% loss of their Fc receptors, a significant decrease in the expression of the C-type lectin heterodimer CD94/NKG2A, a marginal decrease in the Fc receptor associated signaling molecule CD3zeta, and no change in the expression of the tyrosine kinases p56lck and ZAP70 that are involved in lymphocyte activation, and phospholipase C-gamma. CA125 is an equally potent inhibitor of the interleukin-2 stimulated NK cell responses. This phenotype of NK cell exposed to CA125 resembles their corresponding cells isolated from the ovarian tumor microenvironment. Therefore exposure to CA125 may result in the induction of NK cell tolerance during ovarian tumor development *in vivo*. This effect could be crucial for tumor cell escape from the major cell mediated responses. EOC cells, like many other tumor cells, downregulate the expression of major histocompatibility (MHC) class I molecules, thus enabling them to evade responses mediated by class I restricted cytotoxic T lymphocytes (CTLs). However, this strategy should make these cells more vulnerable to cytolysis by NK cells, which lyse tumor cells lacking MHC class I expression. Therefore downregulation of MHC class I expression coupled to NK cell suppression mediated by glycoconjugates like CA125 could enable EOC cells to escape the major cell mediated responses. Anti-CA125 based immunotherapies substantially increase the survival times of EOC patients. Such intervention may prevent CA125 from exerting its immunosuppressive effects thereby leading to an increased capacity of the NK cells to contain ovarian tumors. This ability to suppress NK cell function may also be important during the early stages of human implantation. NK cells constitute 70-80% of the immune cells expressed in the human uterus. CA125 is upregulated in the uterus in the menstrual cycle just prior to the optimal window of implantation. Syncytiotrophoblasts at the leading edge of the implanting embryo also lack MHC class I antigens. The temporal and spatial expression of CA125 during pregnancy suggest that this mucin could also play an important functional role in protecting the embryo during this crucial phase of human development. [This work was supported by the Jeffress Trust and Elsa U. Pardee Foundation (to M.S.P.), the National Institutes of Health (HD35652 to G.F.C), and by the Biotechnology and Biological Sciences Research Council and the Wellcome Trust (to A.D. and H.R.M.)].

(40) **Frontal Affinity Chromatography/Mass Spectrometry in the Screening of Mixtures: Towards Determining the Binding Constant of an Undetectable Compound of Unknown Concentration**

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Frontal affinity chromatography, with detection of small molecule ligands by electrospray mass spectrometry (FAC/MS) has been shown to be a valuable tool for estimating the binding constants of individual compounds present in a mixture. This technique has been applied to quantitatively analyze the binding of oligosaccharides to immobilized lectins, selectins, antibodies, glycosidases and glycosyltransferases. (Angew. Chemie 37, 3384-3387 (1998). Anal. Biochem., 299, 173-182 (2001), Glycobiology 11, 141-147 (2001), Comb. Chem. High Throughput Screen. 5, 395-406 (2002). Mixtures of 100 compounds have been employed.

FAC/MS is currently being used to evaluate mixtures of glycosyltransferase inhibitors produced by solid-phase combinatorial chemistry. The finding that differences in the concentration of ligands does not affect their order of elution over a wide concentration range means that you do not have to know a compound's concentration in order to compare it's Kd value to that of a reference standard. Furthermore, in appropriate mixtures, it is possible to see the effect of unknown and undetectable compound on the retention profile of a reference standard.

(41) **Lessons From Nature: Antibiotic Optimization via Glycorandomization**

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In nature, the attachment of sugars to small molecules is often employed to mediate targeting, mechanism of action, and/or pharmacology. As an alternative to pathway engineering or total synthesis, we report merging two enzymes with upstream synthetic chemistry and downstream chemoselective ligation provides a powerful method (in vitro glycorandomization or IVG) to diversify the glycosylation patterns of complex natural products. Vancomycin glycorandomization revealed the first monoglycosylated vancomycins which rival vancomycin in both activity and scope. The implications for general therapeutic development will also be discussed.

(42) **Chemical Approaches to Studying Protein Glycosylation**

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A major lesson from eukaryotic genome sequencing projects is that the absolute number of genes an organism's genome encodes is not the best parameter for defining complexity of function. It appears that the complex functions associated with human health and disease are determined by combinatorial expansion of genomic information in the form of posttranslational modifications. Of these, the most complex and ubiquitous is glycosylation, highlighting the importance of glycobiology in the postgenomic era. This presentation will focus on new chemical approaches for perturbing and studying oligosaccharide function within the context of the cell surface.

(43) **A Novel Technology for Detection and Proteomic Analysis of O-GlcNAc Modified Proteins**

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The glycosylation of serine and threonine residues by O-GlcNAc is abundant in all higher eukaryotes and has been implicated as an important modification in the regulation of multiple cellular functions. The development of highly sensitive, specific, and efficient methods for the detection, quantification, and proteomics analysis of O-GlcNAc modified proteins would expedite the functional characterization of the modification. Global analysis of O-GlcNAc-modified proteins by proteomics methods is a potentially powerful approach for the molecular characterization of the dynamic modifications in response to a change of cellular environment. However, extant proteomics methods, either 2D-gel/mass spectrometry or ICAT/mass spectrometry-based proteomics methods, are limited to identifying a few thousand of the most abundant proteins and, therefore, are not able to identify low abundance O-GlcNAc modified proteins. To do so would require an enrichment technology that can remove non-O-GlcNAc modified proteins and reduces the complexity of the protein mixture. Here we report a novel technology for the detection, isolation, and subsequent proteomics analysis of O-GlcNAc modified proteins. The technology involves the introduction of a synthetic GlcNAc analog as a replacement for the natural substrate in cellular pathways. The resulting O-GlcNAc modified proteins can then be affinity-purified through a specific conjugation reaction (Staudinger reaction) using a phosphine capture reagent linked to photo-cleavable beads. The captured proteins can then be released by UV light-induced photocleavage. Since affinity purification relies on covalent bonding resulting from a specific and efficient conjugation reaction, other proteins without O-GlcNAc modification can be effectively eliminated by stringent washing. Thus, the technology will allow O-GlcNAc modified proteins to be isolated with high specificity and low contamination. Acknowledgements: YZ is supported by The Robert A. Welch Foundation (I-1550) and NIH (CA 85146). JRF is supported by NIH (GM31278) and the Robert A. Welch Foundation. ML is supported by NIH (GM38545) and Welch Foundation Grant (I-1168).

(44) **Glycosyltransferase Catalyzed Synthesis of Thiooligosaccharides**

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Oligosaccharide analogues in which the interglycosidic oxygen atom(s) have been replaced with sulfur exhibit stability towards glycosylhydrolases and mimic the conformation of the naturally occurring *O*-linked saccharide. As a result of their metabolic stability, thiooligosaccharides are valuable probes of the roles of carbohydrates in a variety of biological phenomena. Thiooligosaccharides have commonly been prepared by chemical means, most frequently by S_N2-type displacement of halide or sulfonate leaving groups by sugar-thiolates.

We report here a chemoenzymatic synthesis of thiooligosaccharides, employing chemically synthesized carbohydrate-thiols as acceptors for glycosyltransferases. Our intial efforts have focused on the use of a 3′-thiolactoside (**1**) as an acceptor. Treatment of **1** with UDP-Gal and recombinant bovine α-(1,3)-galactosyltransferase unexpectedly afforded a linear thio-linked tetrasaccharide. This product was formed by an initial glycosyl transfer to the thiol, and subsequent galactosylation of the terminal α-linked galactose residue. To our knowledge, this represents the first example of glycosyltransferase catalyzed synthesis of thiooligosaccharides. Owing to the relative stability of the thioglycosidic linkage within the tetrasaccharide, the trisaccharide target Gal-α(1-3)[S]-Gal-β(1-4)Glcβ-OR was available as the sole product following incubation with an α-galactosidase from green coffee bean. The rate of glycosyl transfer to the thiol **1** was found to be approximately 100-fold slower than to the native acceptor. This is due primarily to a reduction in V_{max} rather than an effect on substrate binding (K_M). β-(1,3)-N-acetylglucosaminyltransferase has also been shown to catalyze the glycosylation of acceptor **1.** Attempts to utilize other enzymes, including the blood group A and B transferases to create thioglycosidic bonds will be reported.

(45) **Chemoenzymatic Synthesis of Glycosaminoglycans with *Pasteurella* Synthases**

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Glycosaminoglycans [GAGs], linear polysaccharides composed of disaccharide repeats containing a hexosamine, have a wide variety of biological effects and are currently utilized in many medical applications. Sources of very defined uniform GAG polymers, however, are not readily available. We have developed chemoenzymatic methods to produce a variety of hyaluronan [HA], chondroitin, and mixed polymers. We have produced 10 kDa to 1.5 MDa polymers with very narrow size distributions as well as monodisperse short oligosaccharides ranging from 5 to 22 sugars long.

The *Pasteurella multocida* GAG synthase enzymes, pmHAS and pmCS, catalyze the synthesis of HA or chondroitin polymer, respectively, utilizing monosaccharides from UDP-sugar precursors. The synthases will also elongate exogenously supplied GAG oligosaccharide acceptors *in vitro*. We constructed monodisperse GAG polymers of a desired size by controlling reaction stoichiometry (*i.e.* ratio of precursors and acceptor molecules). The polymerization process is synchronized in the presence of acceptor thus all polymer products are very similar. In contrast, without the use of an acceptor, the polymer products are polydisperse. In our syntheses, each specific size class had polydispersity values in the range of ~1.01 for polymers up to ~0.5 MDa or ~1.2 for polymers of ~1.5 MDa (1 is the ideal monodisperse size distribution). We found that both pmHAS and pmCS enzymes will use HA or chondroitin or chondroitin sulfate acceptors. Therefore, we took advantage of this relaxed acceptor specificity to make novel mixed polymers containing more than one type of GAG.

We mapped the two separate active sites of pmHAS and pmCS responsible for adding either the glucuronic acid or the N-acetylhexosamine monosaccharides. We then created two single-action glycosyltransferases (a glucuronic acid-transferase and a N-acetylglucosamine-transferase) from pmHAS by mutagenesis. The pmCS was converted into a single-action N-acetylgalactosamine-transferase. The resulting three enzymes were purified and immobilized individually onto solid supports in an active state. The

glucuronic acid-transferase and a N-acetylglucosamine-transferase enzyme reactors were used in an alternating fashion to produce extremely pure HA polymers of a single length in a controlled, stepwise fashion without purification of the intermediates. The number of reactor steps dictates the final sugar product size. We have made monodisperse HA molecules up to 22 sugars long. If the pmCS-derived N-acetylgalactosamine-transferase reactor is substituted for the pmHAS-derived reactor in certain steps of the process, then novel mixed sugars are produced.
The use of a modified acceptor in either the polysaccharide or the oligosaccharide schemes allows the synthesis of GAG polymers containing radioactive, fluorescent, enzyme or affinity tags. Overall, these reagents should assist in the elucidation of the numerous roles of GAGs in health and disease due to their monodisperse size distributions and defined compositions.

(46) **Syndecans Control Matrix Deposition, Substrate-Dependent Cell Migration and Cell Non-Autonomous Left-Right Signaling**

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During gastrulation in Xenopus, mesoderm cells involute and directionally migrate on the basal surface of embryonic ectodermal cells ("animal cap") and a fibronectin-rich matrix. Later in development, progeny of mesoderm cells give rise to the heart and gut, which display highly conserved Left-Right (LR) asymmetries. We are studying the roles of the transmembrane proteoglycans syndecan-1 and syndecan-2 that are expressed in ectoderm during gastrulation. Syndecan-2 transmits Left-Right (LR) information from ectoderm to migrating mesoderm cells during gastrulation, through a cell-non-autonomous TGFβ-dependent pathway. Syndecan-2 is phosphorylated in right ectodermal cells and not in left ectodermal cells, via a Protein Kinase C-gamma (PKCγ)-dependent pathway. Both phosophorylation states of syndecan-2 are necessary for normal LR development. This raises the question of how a PKCγ-dependent phosphorylation of the cytoplasmic domain of Syndecan-2 controls cell nonautonomous signaling to migrating cells that contact the extracellular domain of syndecan-2. Although Syndecan-2 and Syndecan-1 are expressed in the same ectodermal cells, syndecan-1 does not appear to play a role in LR cell signaling. Specific glycosaminoglycan (GAG) addition sites on the extracellular domain of syndecan-2 are necessary for LR development, others are dispensable. This suggests that the extracellular domains and their glycosylation states are functionally distinct, and that these distinctions along the LR axis are mediated in part by the phosphorylation states of syndecan-2's cytoplasmic domains.
One of the striking aspects of gastrulation is that a fibronectin (FN) matrix is deposited on the "roof" of the blastocoel cavity, on the basal surface of ectoderm cells, and that mesoderm cells migrate on this surface. In contrast, the "floor" of the blastocoel does not have a FN matrix (even though FN monomers are available in the blastocoel cavity) and is not a substrate for directed cell migration. By targeted manipulations of genes in the embryonic cell lineages that give rise to either the roof or the floor, without altering the migratory mesoderm cells, we have developed assays for cell-autonomous matrix deposition and cell non-autonomous control of cell migration. We find that perturbation of endogenous syndecan-1, syndecan-2, β-1-integrin, or the cytoplasmic linker protein PINCH, can block FN deposition on the roof. In the complementary floor assay, de novo expression of syndecan-2 or PINCH alone does not induce FN deposition, but co-expression of both syndecan-2 and PINCH induces FN deposition on the floor and results in re-direction of mesoderm cell migration from its normal path along the roof to a de novo path along the blastocoel floor. These results, along with extensive biochemical analyses of protein interactions, suggest that a previously undescribed complex of syndecan-2, PINCH, and β-1-integrin functions to drive FN fibrillogenesis and to build a substrate that can determine the direction of mesodermal cell migration.

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(47) **Egg Carbohydrates Inducing the Acrosome Reaction in Sea Urchin Sperm**

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Animal sperm cells must undergo the acrosome reaction (AR) to be capable of fertilizing eggs. In the sea urchin, the AR consists of the exocytosis of the acrosomal vesicle (AV) at the anterior tip of the cell and the polymerization of actin to form the 1um long acrosomal process. AR induction is triggered by the influx of Ca++ and Na+ and the efflux of K+ and H+. The increase in Ca++ is through two distinct channels and drives the exocytosis of the AV. The first channel opens in 1s and is dihydropyridine sensitive. The second channel opens 4s later and is sensitive to Ni++. Normally, the first channel activates the second channel. The sperm ion channels are activated by the binding of egg jelly carbohydrates to sperm surface receptors. A 1,000 kDa fucose sulfate polymer (FSP, a pure carbohydrate) in egg jelly is the indispensable species-specific AR inducer. Species-specificity of FSP is determined by the glycosidic linkage and pattern of sulfation. Most species have –1,3-linked fucose and the few species with –1,4-linked FSP are non-inductive in species with –1,3-linked FSP. If FSP is degraded from ~1000 to ~60 kDa, it fails to open the first channel, but does open the second Ca++ channel. The final intracellular concentration of Ca++ can be equal in both forms of FSP, but only the 1000 kDa form triggers the complete AR. Beta-elimination of egg jelly followed by DEAE fractionation yields a polysialic acid (PSA) that greatly potentiates the FSP-induced AR. The PSA increases intracellular pH without increasing intracellular Ca++. The PSA is sensitive to mild periodate and neuraminidase treatment. PSA has no AR inducing activity in the absence of FSP. The PSA probably binds to an unknown sperm receptor. At lease one sperm surface receptor, REJ1, binds to FSP. Monoclonal antibody to REJ1 induces the AR and competes with FSP in AR assays. Three different sea urchin sperm REJ proteins have been cloned. They all have carbohydrate recognition domains of the C-type lectin variety. REJ1 and REJ3 are extracellular disposed, whereas available evidence suggests that REJ2 is an intracellular plasma membrane protein. REJ1 and REJ3 are present on the plasma membrane over the sperm AV. A 1000 amino acid module, "the REJ module", is shared between the three sea urchin REJ proteins and the human polycystic kidney disease proteins polycystin-1 and PKDREJ. Available data suggest that REJ proteins are in someway regulating sperm ion channels. This work connects human disease proteins with the process of signal transduction during the fertilization reaction of a marine invertebrate.

(48) **Bt Toxin v. Worms: It All Comes Down to Sugars**

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Crystal (Cry) proteins made by the bacterium Bacillus thuringiensis (Bt) are pore-forming toxins that specifically target insects and nematodes and are used around the world to kill insect pests. To better understand how pore-forming toxins interact with their host, we screened for C. elegans mutants that resist Cry protein intoxication. We have shown that Cry toxin resistance involves the loss of four glycosyltransferase genes, bre-2, bre-3, bre-4 and bre-5. The bre-2, bre-3, bre-4, and bre-5 glycosyltransferases function in the intestine to confer susceptibility to toxin and are required for the interaction of active toxin with intestinal cells, suggesting they make an oligosaccharide receptor for toxin. The bre-3 gene is the C. elegans homologue of the Drosophila egghead (egh) gene while the bre-5 gene has homology to Drosophila brainiac (brn). These identities are striking given that egh-brn function in a single genetic pathway for epithelial morphogenesis of Drosophila follicle cells. Indeed, we have shown that bre- 3 and bre-5 act in a single pathway in C. elegans with regards to Bt Cry protein intoxication. bre-2 and bre-4 are also part of this pathway. Collaborations with the Reto Muller and in the Hennet Laboratory and Jillian Brown in the Esko Laboratory have confirmed that bre-5 is the C. elegans equivalent of brn. The glycosyltransferase activities of Drosphila EGH and BRN in Drosophila implicate a role for these proteins in glycosphingolipid synthesis. We will update our biochemical and molecular progress in characterization of the glycan that the toxin binds to, our progress in characterizing the receptor that the glycan is attached to, and our progress in cloning the bre-1 gene, a fifth gene implicated in this process.

(49) **Regulation of Notch Signaling by O-Fucose Glycans**

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The Notch gene encodes a receptor protein that mediates a wide range of cell fate decisions during animal development. Notch is modified by two glycosyltransferases that act specifically on EGF repeats, Protein O-fucosyltransferase 1 (OFUT1) and Fringe. We have been using genetic studies in Drosophila, and cell based and in vitro ligand-receptor binding assays, to investigate requirements for Notch glycosylation, and to elucidate the

mechanisms by which Notch glycosylation modulates Notch receptor activation. Both RNAi and mutation of Ofut1 in Drosophila result in Notch loss-of-function phenotypes. The requirement for Ofut1 is cell autonomous, in the signal-receiving cell, and molecular and genetic epistasis experiments position this requirement upstream of Notch activation. Furthermore, binding assays reveal that downregulation of Ofut1 in Notch-expressing cells inhibits both Delta-Notch and Serrate-Notch binding. These results demonstrate that OFUT1 is a new core component of the Notch pathway, which is essential for the activation of Notch by its ligands. Interestingly, over-expression of OFUT1 also influences Notch signaling, but it appears to differentially affect the interaction of Notch with its two Drosophila ligands – potentiating Serrate-Notch interactions, but inhibiting Delta-Notch interactions. These effects are opposite to, and are suppressed by, the β-1,3-N-acetylglucosaminyltransferase encoded by the fringe gene, which modulates Notch signaling by inhibiting the activation of Notch by Serrate and potentiating the activation of Notch by Delta. Like OFUT1, Fringe can also influence Notch activation at the ligand binding step. Subsequent to action of Fringe, in CHO cells Notch1 is elongated by a β-1,4-Galactosyltransferase and a Sialyltransferase. We have examined the consequence of mutation or RNAi of each of the three predicted β-1,4-GalTs in Drosophila, and are currently examining double mutant combinations. To understand how O-fucose glycans regulate Notch signaling, we also need to identify the biologically relevant sites of glycosylation. To do this we are making mutations in predicted O-fucose sites of Notch and its ligands. A revised, broad consensus sequence for O-fucosylation predicts that up to 23 of the EGF repeats in Drosophila Notch, 8 of the EGF repeats in Serrate, and 7 of the EGF repeats in Delta may be modified by O-fucose. Mutation of all 8 sites in Serrate, and all 7 sites in Delta, reveals that O-fucosylation is not required for its ability to function as a Notch ligand or to be regulated by Fringe. Mutation and in vivo analysis of O-fucose sites in Notch is in progress. A highly conserved O-fucose site in an EGF repeat that is critical for ligand binding, EGF12, is not essential for Notch activation, but is essential for effective inhibition of Notch-Serrate interactions by Fringe. We have also identified a series of O-fucose sites in EGF repeats in Notch that appears to be essential for Notch activation, but not for ligand binding.

(50) **Chondroitin Is Required for *C. elegans* Vulval Development and Embryogenesis**

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A previous screen for *C. elegans* mutants defective in vulval invagination identified eight *squashed vulva* (*sqv*) genes. In *sqv* mutant L4 larvae the vulval extracellular space fails to expand and the vulva appears collapsed. Stronger *sqv* mutations also show maternal effect lethality as a result of the inability of the progeny of homozygous mutants to initiate cytokinesis at the first cell division. Characterization of seven *sqv* genes showed they define components of the glycosaminoglycan biosynthesis pathway. Three of these genes generate or transport nucleotide sugar precursors: SQV-4 is the UDP-glucose dehydrogenase that catalyzes formation of UDP-glucuronic acid; SQV-1 is a decarboxylase that synthesizes UDP-xylose; and SQV-7 is a multi-transporter that imports three nucleotide sugars into the Golgi apparatus. Studies of SQV-6, SQV-3, SQV-2, and SQV-8 identified them as xylosyltransferase, galactosyltransferase-I, galactosyltransferase-II, and glucuronosyltransferase-I, respectively. These enzymes are required for the assembly of the proteoglycan core protein linkage tetrasaccharide GlcAβ1,3Galβ1,3Galβ1,4Xylβ-O-(Ser), which is common to the two major types of glycosaminoglycans, chondroitin and heparan sulfate. We report that the eighth *sqv* gene, *sqv-5*, is the *C. elegans* ortholog of the chondroitin synthase involved in polymerization of the chondroitin chain. Biochemical assays showed substantial synthase activity in wild-type worms, approximately one-half of that activity in *sqv-5* heterozygotes, and virtually no activity in homozygous mutants. In contrast, activity levels of the heparan sulfate co-polymerase were comparable in wild-type and *sqv-5* mutant worms. Chemical analyses demonstrated that *sqv-5* mutants have negligible chondroitin levels, while wild-type worms contain 180 fmol/worm. Western blots of partially purified proteoglycans showed that worms treated with *sqv-5* RNAi had a reduction in chondroitin-containing proteoglycans, while levels of heparan sulfate proteoglycans remained constant. Because the phenotype of the *sqv-5* mutant appears to be identical to that of the other *sqv* mutants, these data suggest that vulval and embryonic defects are a consequence of a lack of chondroitin, not heparan sulfate, in the developing animal. Studies are underway to identify the major proteoglycan core protein involved in these processes.

(51) **Trans-Cellular Induction of Neural-Specific Glycosylation by A Toll-Like Receptor**

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Complex carbohydrate expression in developing tissues is temporally and spatially regulated. The presentation of specific oligosaccharide structures by appropriate cell types at appropriate developmental stages is required for normal differentiation and morphogenesis. As a consequence of their regulated expression, glycans frequently serve as useful markers for specific cell-types. For instance, a family of N-linked oligosaccharides, collectively known as the HRP-epitope, is found at the surface of all neurons in the embryonic nervous system of many arthropods, including *Drosophila melanogaster*. We have utilized a *Drosophila* mutation, that results in loss of the neural HRP-epitope, to identify components of the pathway leading to restricted glycan expression. Previous molecular characterization of the mutation determined that the affected gene encodes a transmembrane protein of the Toll-like receptor family (TLR), which we designated "Tollo." Further analysis demonstrates that *tollo* is not transcribed in neurons, despite the fact that loss of Tollo abolishes expression of the HRP-epitope in neural cells. Rather, *tollo* mRNA is detected in ectodermal cells that surround differentiating neurons prior to expression of the HRP-epitope. To assess the cellular mechanism by which *tollo* induces specific glycosylation, we have used the Gal4/UAS system to drive expression of Tollo in specific cell populations within embryos that otherwise lack the *tollo* gene. When Tollo expression was driven by its own promoter, neural HRP-epitope was rescued in the *tollo* null background. Neither a pan-neural driver (*ELAV*-Gal4) nor a mesectodermal/midline glial driver (*rhomboid*-Gal4) rescued oligosaccharide expression, despite their ability to drive Tollo expression in cells that make extensive contact with neuronal surfaces. Therefore, simply presenting Tollo protein to neurons is insufficient; induction of the neuronal HRP-epitope requires Tollo expression in appropriate non-neural ectodermal cells. More generally, our misexpression results indicate that TLR-induced glycosylation requires the generation of a second intercellular signal following TLR activation. Interpretation of the propagated signal by receptive cells in the local environment leads to tissue-specific glycan expression.

(52) **Roles of O-Fucose Glycans in Ligand Binding to Mammalian Notch Receptors**

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Notch receptor 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 b1,3-*N*-acetylglucosaminyltransferases. Moreover, the addition of Gal by b1,4Galactosyltransferase-1 to GlcNAc added by Fringe is required for Jagged1-induced Notch signaling. We are investigating the mechanisms by which *O*-fucose glycans modulate Notch signaling. Binding of soluble Notch ligands conjugated to the Fc region of human IgG (rat Jagged1-Fc and rat Delta1-Fc) to endogenous Notch receptors expressed on the cell surface was measured by FACS analysis. Embryonic stem (ES) cell lines derived from mouse blastocysts lacking O-FucT-1 or from wild type blastocysts were cultured without feeder cells in the presence of leukemia inhibitory factor (LIF), trypsinized, cultured for 5 hours in suspension, and assayed for ligand binding. Both Jagged1-Fc and Delta1-Fc bound to Pofut1$^{+/+}$ ES cells and binding was inhibited by the addition of EDTA, indicating that binding was specific to Notch. By contrast, neither Jagged1-Fc nor Delta1-Fc bound to Pofut1$^{-/-}$ ES cells. However, the level of cell surface expression of Notch1 and Notch2 receptors was similar in Pofut1$^{+/+}$ and Pofut1$^{-/-}$ cells. Therefore, *O*-fucosylation by *O*-FucT-1 appears to be required for ligand binding to Notch, but not to be required for cell surface expression of Notch receptors. Similar experiments are being performed in CHO cells and CHO glycosylation mutants expressing Fringe to identify the role of each sugar of the *O*-fucose tetrasaccharide on mammalian Notch receptors in Notch ligand-receptor binding.

(1) Shi, S. and Stanley, P. Proc. Natl. Acad. Sci. USA 100, 5234-5239, 2003.

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(53) **Glycobiology of Neuronal Regeneration in the Adult CNS**

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The adult mammalian central nervous system (CNS) is a profoundly inhibitory environment for axon regeneration. This is due, in significant measure, to multiple axon regeneration inhibitors (ARI's) in the milieu of a CNS injury. These include Nogo, myelin-associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (OMgp) and chondroitin sulfate proteoglycans (CSPG). Each ARI binds to complementary ligands on the nerve or axon surface, halting axon regeneration. Rapidly emerging knowledge of ARI's and their ligands provides previously unanticipated opportunities to block their actions and potentially enhance axon regeneration.

Each ARI and/or its nerve cell surface ligand(s) are glycosylated. Moreover, glycosylation plays key structural and functional roles in inhibiting axon regeneration. The nerve cell ligand for Nogo, NgR, is a glycosylphosphatidylinositol (GPI)-linked glycoprotein, as is OMgp. MAG is a sialic acid binding protein that binds to nerve cell surface gangliosides GD1a and GT1b, as well as to NgR. Finally, the sugar chains of CSPG mediate its inhibition of axon regeneration. The tools of glycobiology provide feasible means to block ARI's and enhance axon regeneration.

Our laboratory has focused on the role of myelin-associated glycoprotein (MAG) in nerve regeneration. MAG, a member of the Siglec (sialic acid binding/Ig-superfamily/lectin) family, binds preferentially to glycans bearing the NeuAcα3Galβ3GalNAc non-reducing terminus. These include the most prominent sialylated glycans in the adult brain, gangliosides GD1a and GT1b. Support for the role of these gangliosides as physiological MAG ligands derives from nerve regeneration studies in vitro. Newborn rat cerebellar granule cells elaborate an extensive network of neurites on control surfaces, but not on surfaces with MAG. The inhibition of neurite outgrowth by MAG is reversed by blocking glycosphingolipid biosynthesis and by antibodies to GD1a and GT1b. Furthermore, MAG inhibition is mimicked by specific multivalent crosslinking of these gangliosides.

Studies on the carbohydrate binding specificity of MAG for gangliosides revealed that rare "alpha-series" gangliosides, which carry the non-reducing terminus NeuAcα3Galβ3(NeuAcα6)GalNAc were even more potent MAG binding ligands than GD1a or GT1b (Collins, B.E., et al. (1999) J. Biol. Chem. 274, 37637). Recent work (Blixt, O., et al (2003) J. Biol. Chem. 278:31007) further discovered that a synthetic threonine glycoside of the related α3/α6 disialylated T antigen (NeuAcα3Galβ3(NeuAcα6)GalNAcαThr(OCH3)) was a highly potent monovalent inhibitor of MAG in a multivalent binding assay. Based on that study, we tested the ability of α3/α6 disialylated T antigen and related monovalent glycans to reverse MAG inhibition of neurite outgrowth. The above glycoside reversed MAG-mediated inhibition of neurite outgrowth with a half-maximal potency of ~10 micromolar, whereas related and control structures were significantly less potent or ineffective. These data support the exploration of monovalent MAG-binding glycans to enhance nerve regeneration, and further demonstrate the potential for glycobiology to impact the study of neuronal plasticity in the adult CNS.

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(54) **Polysialic Acid and Neural Plasticity**

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The polysialylated form of the neural cell adhesion molecule (PSA-NCAM) appeared during the evolution of vertebrates as a new mechanism for regulation of cell interactions. This large and abundant glycoprotein can exert steric effects at the cell surface that lead to the attenuation of cell-cell bonds mediated not only by NCAM but also a variety of other adhesion receptors. PSA-NCAM expression changes both as a result of developmental programs and physiological inputs. This global modulation of cell-cell attachment has been shown to facilitate cell migration, axon pathfinding and targeting in the embryonic. The much more restricted expression of PSA in the adult nervous system has been found to reflect the capacity of these tissues to undergo plastic changes in structure and function, as evidenced in circadian rhythms, responses to chronic pain and stress, and adult neurogenesis. Alteration of tissue plasticity can also be induced by viral delivery of the PST polysialyltransferase, and this procedure may serve to improve recovery of neural tissues from injury.

(55) **Broad Yet Surprisingly Specific Functions of Heparan Sulfate in Mammalian Brain Development**

Yu Yamaguchi
The Burnham Institute

No abstract available

(56) **Glycosylation and Dystroglycan Function in Muscular Dystrophy**

Kevin Campbell
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No abstract available

(57) **Hereditary Inclusion Body Myopathy; Epimerase Activity, *GNE* Mutations and Treatment Strategies**

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Hereditary inclusion body myopathy (HIBM) is an autosomal recessive disorder characterized by adult-onset, progressive distal and proximal muscle weakness sparing the quadriceps muscle. Homozygosity mapping of a Middle Eastern Jewish isolate allowed identification of the gene underlying this disorder, *GNE*, which encodes the enzyme, uridine diphosphate-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase or GNE. GNE is the bifunctional and rate-limiting enzyme involved in the biosynthesis of sialic acid (N-acetylneuraminic acid). Mutations in either or both the epimerase and kinase domains of GNE have been reported to cause HIBM. The epimerase converts UDP-GlcNAc to N-acetylmannosamine (ManNAc) and the kinase converts ManNAc to ManNAc-6-phosphate, precursors in the sialic acid synthetic pathway. We assayed GNE epimerase activity using UDP[^{3}H]GlcNAc as substrate and measuring the product, [^{3}H]ManNAc in cultured skin fibroblasts from three patients with HIBM. Patient #1, with two epimerase domain mutations (G455T and C787T), had 38% of control fibroblasts epimerase activity (n=2). Patient #2, with one epimerase mutation (T698C) and one kinase mutation (C1943T), had epimerase activity 48% of control (n=4). The third patient (#3), with homozygous kinase mutations (T2186C) consistent with the Middle Eastern Jewish isolate, had epimerase activity 83% of control (n=4). These findings indicate a rough correlation of epimerase activity with the number of GNE epimerase domain mutations. Sialic acid modification of cell surface glycoproteins are crucial for cell adhesion and signal transduction. Defective glycoconjugate sialylation in a patient with HIBM was demonstrated by using the lectin WGA-FITC, which recognizes terminal sialic acid of oligosaccharides on cellular glycoproteins. In normal fibroblasts, immunofluorescence showed a punctate pattern throughout the cells, however, in HIBM fibroblasts, there was only residual staining around the Golgi. When growing the patient's cells in media containing free sialic acid or ManNAc, the WGA-FITC fluorescence was corrected to a normal pattern. These findings suggest that supplementation with ManNAc or free sialic acid may be a therapeutic intervention for patients with HIBM.

(58) **Amylose, Chitin and the Glycopathobiochemistry of Alzheimer's Disease: A Reevaluation of the Origin, Composition and Significance of Amyloid Plaque.**

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Here we present the first report of the presence of β-linked polymers of N-acetylglucosamine (chitin) and amylose in brain tissue of patients who suffered from Alzheimer's disease and indeed the first report of the presence of chitin in humans. The search for chitin was prompted by the discovery of high levels of soluble β-linked glucosamine-rich glycans in tissue from Alzheimer's diseased brains indicating that this insoluble glucosamine-containing polymer might also be present. Fibrils in senile plaques and blood vessels stained with calcofluor, a carbohydrate-specific stain used especially for the detection of β-1,4-linked carbohydrate polymers such as cellulose and chitin. Calcofluor is not known to stain proteins. Actual fibrils were isolated from AD brain after exhaustive digestion of tissue with DNAse, RNAse and amylases followed by proteases. They were characterized by Fourier transform infrared spectroscopy on individual fibers using IR-microscopy. Compositional analysis using GC-MS confirmed glucosamine as the only component. Enzymatic degradation with chitinase yielded chitobiose. The isolated fibers and authentic chitin both stained identically with congo red displaying the same characteristic yellow to

yellow green birefringence as do amyloid plaques. These results show that chitin forms a key component in the insoluble Alzheimer's plaque matrix which is also known to contain several proteins and might be the basis for extreme insolubility of Alzheimer's plaque. The presence of new soluble glucosamine-rich glycans in the diseased state also has implications for immune responses. These findings open several new avenues for strategies for developing pharmaceutical therapies for Alzheimer's disease.

(59) **Region-Specific and Epileptogenic-Dependent Expression of α2,3-Sialyltransferase in the Adult Mouse Brain.**

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Brain is formed based on the complex neural circuits, in which neural plastic changes induce modification of the circuits and appearances of the new roots. Neural plasticity needs diversity and individuality, while fundamental molecules, such as channels, receptors, and structural molecules containing cytoskeleton, are essential for brain function, not having variation. Alternatively, glycosylation is one of the most familiar post-translational modifications providing variation to the fundamental molecules. Thus, we have investigated whether glycosylation affects acquirement of neural plasticity. First, we selected amygdaloid kindling mouse model to observe neural plasticity changes in *in vivo* brain. The kindling is a model of human temporal lobe epilepsy, in which excitability in limbic structures is permanently enhanced by repeated stimulation. We confirmed that behavioral, physiological, and dendritic aberrations gradually occurred in mice during kindling-epileptogenesis. Briefly, adult mice received a biphasic square wave pulse [495 μA, 60Hz, 200 μS duration, for 2 sec] unilaterally in the basolateral amygdaloid complex once a day. Acquirement of kindling-seizures was monitored with behavioral and electroencephalographical 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 is 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. Then we used the kindling to screen differential glycosylation dependent on neural plasticity changes in brain. In brain, large species of glycoconjugates show the sialylated form and compositions of gangliosides are conserved in the most animals. As the sialylated group possesses a negative charge at the terminal position of sugar linkages on protein and lipids, cell-cell interactions may be induced by the charge. Additionally, several reports suggested that several sialylated glycoconjugates, such as PSA on N-CAM and gangliosides, were involved in the long-term potentiation. Thus, we have focused on sialylation among glycosylation. Sugar structures are heterogeneous in various tissues, while the cell population and connections in the brain also show diversity. Therefore, it has been difficult to evaluate unique functions of sialylated glycoconjugates in the brain. Thus, we screened differential expression of sialyltransferase genes. Finally, we found that mRNA and the endo-product of α2,3-sialyltransferase (ST3Gal IV) increased dramatically and gradually in neuronal cells during kindling-epileptogenesis. Especially, the increases were observed in the hippocampus and the anterior thalamic nuclei. The amygdaloid complex connects bilaterally with the anterior thalamus via the hippocampus. Therefore, it was suggested that up-regulation of ST3Gal IV expression was induced by indirect projection of fibers from the site of kindling-stimulation. Finally, these findings provide that ST3Gal IV expressions are regulated by physiological activity and may play a role in neural plasticity.

(60) **Detection and Quantification of Twelve Heparin- and Heparan Sulfate-derived Disaccharides by Electrospray Ionization Ion Trap Tandem Mass Spectrometry: Application to Heparin/Heparan Sulfate Oligosaccharide Sequencing**

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Introduction

The unique sulfated saccharide sequences of heparin/heparan sulfate(HS) glycosaminoglycans (GAGs) are likely what regulates their ability to interact with a variety of proteins, and thus gives rise to the diverse (patho)physiological functions attributed to them, including effects on hemostasis and cell development. Heparin/HS polysaccharides consist of a variably sulfated disaccharide repeating unit (a hexuronic acid α(1→4) linked to a glucosamine unit). The disaccharide may be *N*-sulfated or *N*-acetylated, and further sulfation can occur at the C-2 position of the hexuronic acid as well as the C-6 position of the glucosamine. There are few techniques, including HPLC and CE, that are currently available to separate and characterize heparin/HS oligosaccharides, and therefore the relationship between the saccharide sequences and their diversified biological functions still remains obscure in many cases. This abstract reports a method in which electrospray ionization mass spectrometry (ESI-MS) and MS/MS are used for the identification and quantification of twelve of the constituent disaccharides in heparin/HS, and the method's application to heparin/HS oligosaccharide sequencing.

Methods

Mass spectra were obtained on a quadrupole ion trap instrument using electrospray ionization. Disaccharide standards and mixtures were sprayed at 20 pmol/μL from a 70% MeOH solution containing 10 mM NH_4OH. Samples were introduced by flow injection analysis at 20 μL/min and spectra were obtained in negative ionization mode. Complete depolymerization of heparin/HS samples was carried out by overnight digestion using a mixture of heparin lyases I, II and III. Samples were then analyzed by ESI-MS/MS without further purification. In addition, intact heparin/HS oligosaccharides were sprayed at 20 pmol/μL using direct infusion and analyzed via MS/MS and MS^3 to determine sequence.

Results

This development extends our previous method to include the identification and quantification of twelve heparin/HS-derived Δ-disaccharides obtained by enzymatic depolymerization.[1] The protocol was applied to the compositional analysis of five biologically isolated heparin/HS samples from porcine and bovine sources, and the results were found to be in accordance with those previously reported. The analysis is rapid, accurate and uses no purification or separation steps prior to analysis by MS, thus reducing sample consumption and analysis time of traditional methods. The twelve disaccharide constituents could all be identified from a combination of MS^1 and MS/MS, and quantified from full scan MS^1 spectra using an internal standard (I-P), and a calculated response factor for each disaccharide. Diagnostic product ions from MS/MS spectra of isomeric disaccharides IIA/IIIA, IVS/IIH/IIIH and IIS/IIIS/IH were used for the quantification of the relative amounts of each of the eight isomers in mixtures.

The compositional analysis method was also applied to isolated heparin oligosaccharides, and used in conjunction with MS^n data on the intact oligosaccharides, for their sequence determination. Results obtained suggest that the described method can be accurately applied for the determination of disaccharide compositions of unfractionated heparin/HS and low molecular weight heparins (LMWH), as well as to the analysis and sequencing of isolated heparin oligosaccharides from various biological sources.

[1] Saad, O.M.; Leary, J.A. *Anal. Chem.* **2003**, *75*, 2985-2995.

(61) **Glycan Fingerprinting: Two-Dimensional Electrophoresis of Fluorescent-Derivatized Oligosaccharides**

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The separation and analysis of complex mixtures of glycans to allow comparison of oligosaccharides obtained from different sources requires a number of separation steps and is time consuming. Two-dimensional electrophoresis has proved to be a convenient method of high resolving power to separate polypeptides. We report here the use of boronate affinity electrophoresis in conjunction with high density sizing polyacrylamide gel electrophoresis for the 2-D separation of oligosaccharides derivatized with the anionic fluorescent dye, Cascade Blue ethylene diamine derivative or 8-aminonaphthalene-1,3,6-trisulfonic acid. The resolving power and reproducibility of the method is demonstrated with glycan mixtures isolated from ovalbumin and desialylated fibrinogen.

(62) **Towards Understanding O-Glycan Structural Diversity**

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Glycoproteins containing heavily O-glycosylated, mucin-like domains serve important biological functions where the O-linked glycans play major roles. Although poorly understood, O-glycan structures seem to vary reproducibly as

a function of their position in a peptide sequence. To aid in understanding such variation we have recently characterized the *in vivo* glycosylation pattern of the porcine submaxillary gland mucin (PSM) tandem repeat, containing 31 O-glycosylated Ser/Thr residues (Gerken et. al. JBC 277, 7736 (2002)). We found that the peptide GalNAc and Core 1 (R- β-Gal(1-3) α-GalNAc-O-Ser/Thr) structures appeared inversely correlated to the density of the glycosylated Ser and Thr residues. By contrast, the α-(1-2) fucosylation of the Core 1 glycans varied depending on the nature of the peptide-glycan linkage, with Ser linked glycans having higher fucosylation than Thr linked glycans. Recent kinetic modeling of the site specific *in vitro* glycosylation of the PSM tandem repeat by ppGalNAc T1 and T2 has demonstrated the presence of inhibitory neighboring glycosylation effects (Gerken et. al. JBC 277, 49850 (2002)). Studies in progress with ppGalNAc T1 and T2 on the tandem repeat from the canine submaxillary mucin (CSM) further confirm the role of neighboring glycosylation effects and reveal unique peptide sequence preferences for ppGalNAc T2. These findings demonstrate that the ppGalNAc transferases are modulated by both neighboring group glycosylation and by the nature of the peptide sequence. Kinetic modeling has also confirmed the inhibitory effects of neighboring glycosylation on the formation of the Core 1 glycans *in vivo*. Since the model is capable of predicting the *in vivo* Core 1 pattern, it appears that the formation of the Core 1 structure, an important initial step in O-glycan biosynthesis, may be solely regulated on the basis of neighboring residue glycosylation. Studies are also in progress characterizing the α-(2-6) sialylation pattern (by N-glycolylneuraminic acid (NeuNGl)) of the peptide linked GalNAc residues of the PSM tandem repeat. A preliminary analysis suggests that NeuNGl substitution of non-Core 1 (mono) GalNAc residues is unaffected by neighboring glycosylation, and is simply proportional to the mono GalNAc concentration. The determination of the sialylation pattern of the Core 1 structures is in progress. In contrast to the Core 1 transferase, the 2-6 sialyltransferase appears to be insensitive to neighboring glycosylation. Whether these differences solely reside in the nature of the transferases or are due to differences in accessibility of the C3 and C6 GalNAc hydroxyl groups is presently unknown. In summary, we have begun to identify the different substrate factors that influence the activities of the ppGalNAc, Core 1, fucosyl and sialyl transferases involved in initial O-glycan biosynthesis. Our ability to kinetically model the behavior of several of the transferases suggests that the prediction of O-glycan core structures in a site specific manner may become a possibility. Supported by NIH Grant RO1- CA 078834.

(63) **Glycomics of Glycosaminoglycans**

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Proteoglycans represent a class of highly versatile molecules containing recognition markers both on their glycosaminoglycan (GAG) and on their protein moieties. GAGs were shown to interact with growth factors, chemokines, cytokines, matrix molecules, cell membrane receptors, enzymes and clotting cascade components in a highly specific manner. The primary structure of chondroitin sulfate (CS) and dermatan sulfate (DS) oligomers is based on the alternating uronic acid and N-acetylgalactosamine monosaccharide building blocks modified by sulfate groups. In chondroitin/dermatan sulfate GAGs, functionally relevant in interaction with fibroblast growth factor-2 (FGF-2), the segregation into alternating blocks of specific epimerisation and sulfation patterns is determining the oligosaccharide sequences with specific binding functions. We recently extended potentials of modern mass spectrometry (MS) to enable detection and identification of single components in complex GAG mixtures derived from protein-linked glycans. In this context we developed a new type of sheathless interface to couple capillary electrophoresis (CE) to mass analyzers and perform anion mapping by ESI-MS and sequencing by tandem MS. CE/nanoESI QTOF MS demonstrated already high potential for analysis of biological material for peptide- and glycoconjugate screening [1, 2]. The feasibility of this approach for glycomics of GAG oligosaccharides from biological material was explored on CS/DS oligosaccharides obtained from bovine aorta [3] and applied to CS/DS oligosaccharide mixtures obtained from human skin and kidney fibroblast decorin [4]. Applying this basic technology for the first time the limits for glycosaminoglycan sequencing have been extended to large CS/DS oligomer species, containing up to 22 monosaccharide moieties, not detectable before [5].
A second, highly valuable, approach has been developed under the use of high resolution Fourier Transform Ion Cylotron Resonance (FT-ICR) MS at 9.4 T to explore the expression of highly charged overlapping anionic species present in complex GAG mixtures in the negative ion mode nanoESI. Oligosaccharide heterogeneity of the depolymerized CS/DS mixture obtained after chondroitin B lyase digestion of bovine aorta arising from the different size of the sugar chain and from the different degree of sulfation was deciphered using the accurate mass determination better then 1 ppm [6].
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(64) **High-Throughput Functional Affinity Purification of Mannose Binding Proteins from *Oryza sativa***

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Lectins are an essential tool in the structural analysis of carbohydrates. New lectins with different specificity to those currently available are of great interest and potential commercial value. We present here a pilot study using proteomics technologies to annotate potential new lectins in the recently completed draft genome sequence of rice (*Oryza sativa*). We have used affinity chromatography in combination with mass spectrometry to isolate, identify, and assign a preliminary functional annotation to a large number of both known and novel proteins from rice. Rice leaf, root, and seed tissue extracts were fractionated by affinity chromatography over an agarose-linked α-D-mannose column. Bound fractions were eluted and subjected to 1-D electrophoresis, followed by nanoflow high performace liquid chromatography - tandem mass spectrometric analysis of spearated proteins. This multiplexed technology resulted in the isolation and identification of 136 distinct mannose-binding proteins from rice. A comparative analysis demonstrates very little overlap of identified proteins between the respective tissues, and confirms the correctly compartmentalized presence of a significant number of proteins from largely tissue-specific biochemical pathways. Over 30% of the identified proteins having a previously known function are directly involved in sugar metabolism. These numbers include several highly expressed known rice lectins. Comparison of the peptide sequences identified in this study to those peptides identified in the most comprehensive survey of the rice proteome to date indicates that our current data represents a significant enrichment of proteins unique to this dataset. Nearly 15% of the identified proteins, identified on the basis of exact peptide matching to sequences in the rice genomic database, represent proteins without a known functional annotation, indicating the potential of this combined approach to assign a preliminary carbohydrate-binding function to novel proteins in a high-throughput fashion

(65) **Glycoblotting: Synthetic Nanoparticles for Rapid Isolation and Analysis of Glycopatterns**

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A new technique for the rapid analysis of trace amounts of oligossacharides in crude samples is essential in the field of glycobiology and pathology. We have

developed an effective and practical trap-and-release method based on chemoselective ligation of carbohydrates with reactive hydroxylamine groups displayed on the surface of nanoparticles as a scaffold. We have chosen a hydroxylamine-attaching photopolymerizable lipid as an anchor molecule for capturing oligosaccharides. Liposomes of diacetylene-containing lipids can be readily polymerized by UV irradiation to produce polymer-based nanoparticles. The oligosaccharides (N-glycan) were released from human IgG by enzymatic degradation using protease and glycanase. The nanoparticles were mixed with the crude IgG sample for 6 hrs in a buffered solution (pH 5.2). After collection of sugar-trapped nanoparticles by spinfiltration, carbohydrates are released from the particles under an acidic condition. MALDI-TOF mass spectra before purification did not show any significant signals corresponding to N-glycans because these signals were hidden by the large signals due to peptides. However, after purification by the nanoparticles, only the glycopatterns were observed. The signal intensity corresponding to each N-glycan was in good agreement with the ratio of each N-glycan of IgG as revealed by HPLC technique using common PA labeled-sugars. Our approach will greatly simplify and facilitate the process of purification and isolation of trace amounts of sugars without the need for any tedious and chromatographic techniques, providing a novel strategy for high-throughput glycoform analysis in combination with mass spectrometry.

(66) **Discovery of Two New Polysialoglycoproteins in Human and Sea Urchin. Linkage and Length Diversities of Polysialic Acid in Animal Glycoproteins**

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Polysialic acid (polySia) is a linear polymer with a degree of polymerization (DP) greater than 8 Sia residues. Recently we have developed highly sensitive chemical and immunochemical methods to detect not only polySia but also di- and oligoSia (DP 3-7). With these methods, we have demonstrated that several glycoproteins in brain, adipose tissue, and blood have the diSia and oligoSia structures, demonstrating the chain length diversity of polySia as a common feature in animal glycoproteins [1-3]. A further attempt to search for the di/oligo/polySia chains on glycoproteins has led us to the finding of two new members of the polySia-containing glycoproteins.

First, α2,8-linked polyNeu5Ac has been detected in human milk CD36 [4]. The milk CD36 is the third example of the polySia-containing glycoprotein. So far, neural cell adhesion molecule and α-subunit of the voltage-dependent sodium channel in brain have been only two examples of the α2,8-polySia-glycoproteins in mammals. The polysialylated CD36 is unique in that the polySia chain is on its *O*-glycan, while the other two contain the polySia chain on *N*-glycans. Polysialylated CD36 occurs in mammary gland, but not in platelet. The extent of polysialylation of milk CD36 changes depending on lactation stages. These results indicate that polysialylation of CD36 is regulated in a tissue-specific and a lactation stage-specific manners.

Second, the presence of α2,9-linked polyNeu5Ac in a novel sperm flagellum polysialoglycoprotein (SF-PSGP) of sea urchin has been demonstrated. SF-PSGP is a mucin-like glycoprotein and a major sialoglycoprotein in sperm. The polySia chains reside on *O*-glycans, but not on *N*-glycans. Notably, the ketosidic linkages in the polySia structure are exclusively α2→9, but not α2→8. The DP of the polySia chain is on average 15, and is mostly capped by 8-*O*-sulfated Neu5Ac. These results demonstrate there is the linkage diversity of polySia in animal glycoproteins. This is the first example of the occurrence of polySia in animal sperm. Since SF-PSGP is enriched in membrane microdomain (lipid raft) of sea urchin sperm, the polySia may possible involved in regulation of the membrane microdomain-mediated sperm-egg interaction that has been recently shown by us [5].

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(67) **Analysis of Individual Tracheo-Bronchial Mucin O-Glycosylation Profiles**

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In pathological conditions, mucus from airways exhibits altered physico-chemical properties which may have profound physiological repercussions. Indeed, important modifications of O-glycosylation pattern have been reported in cystic fibrosis (CF) bronchial mucus [1]. In particular, an increased sulfation and sialylation that lead to an up-regulation of Sulfo- and Sialo-Lewis x motifs would account for the increased susceptibility of CF patients towards *Pseudomonas aeruginosa* respiratory tract infection [2]. Full understanding of the biological relevance of these observations is undermined by a lack of information concerning both the fine organisation of cell surface and secreted mucins within the non-pathological mucus layers and the glycosylation patterns of their individual components. However, recent advances in mass-spectrometry techniques associated with development of specific protocols aimed at the separation of individual mucin glycoforms has opened up new fields of investigations.

To provide a better insight into the mucin structure of normal epithelia and modifications occurring during airways pathologies, we have purified and characterized intact mucins according to their physico-chemical properties, their location within the mucus layer and their MUC gene origin from both healthy individuals and early-passage normal human tracheo-bronchial epithelial (NHTBE) cell cultures. Some mucins could be further fractionated into distinct glycoforms that presented distinct sulfation patterns on the basis of their chromatographic properties. The identity of each mucin sample was established prior to analysis of the O-glycans through a combination of biochemistry and proteomic methods. O-glycosylation profiles of each purified mucin were then mapped by mass spectrometry techniques, based primarily on the use of the MALDI-QTOF instrument. Characteristic fragmentation patterns established by MALDI-CID MS-MS on a large panel of permethylated purified O-glycans permitted a facile discrimination between closely related glycan motifs. In particular, we took advantage of both linkage-specific loss of fucose residues and specific elimination of C-3 substitutions on GlcNAc to easily identify Lewis type motifs directly from CID MS-MS fragmentation patterns. We further observed a partial desulphation of sulphated O-glycans upon laser desorption that eases both their identification in mixture, and their subsequent sequencing by CID MS-MS. On these bases, we analysed the O-glycosylation patterns of diverse bronchial mucins preparations, first on total O-glycans preparations and then after HPLC separation and mild periodate oxidation. This strategy permitted in a first set of experiments to confirm the extraordinary diversity of mucin-type O-glycosylation, establishing that CF mucus, used as reference material, comprises at least one thousand different O-glycans. Furthermore, it appeared that CF mucus exhibits a much more complex O-glycan profile than total secretions from NHTBE cell cultures, in part owing to the expression of specific motifs such as core 4 (GlcNAc(β1-3)[GlcNAc(β1-6)]GalNAc) structure which is totally absent from cell culture secretions.

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(68) **Structural Assignment of Isomeric 2-Aminopyridine Derivatized Oligosaccharides Using Ms^n Spectral Matching**

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The rapid growing interest in biological role of oligosaccharides and glycoproteins has been resulting in the widespread use of mass spectrometry (MS) for their analysis as well as peptide and protein analysis. However, it is still a big challenge for MS to determine isomeric monosaccharide component, linkage position, anomeric configuration, and branching appearing in a diversity of oligosaccharide structures[1, 2]. So far, the 2-dimensional (2D) or 3-D mapping method[3, 4] has been used for analyzing N-glycans. It is combined high-performance lipuid chromatography (HPLC) analysis using ion exchange, silica-ODS and amide columns, oligosaccharide derivatization by,

e.g., 2-aminopyridine (PA), and partial digestion by acid hydrolysis or sequential exoglycosidase treatments. This traditional 2D or 3D mapping method works well, but it is very time consuming. Our aim is to develop an efficient LC-MS method for oligosaccharide analysis instead of the traditional 2D or 3D mapping method, relying on both a HPLC separation capability and a MS^n spectral database of known oligosaccharides.
Here, we selected two isomeric pairs of 2-aminopyridine derivatized (PA) fucosylated and nonfucosylated N-glycans which definitely yield the same base ion in MS spectra and therefore, are impossible to distinguish them only from their MS spectra. First, a reproducibility of MS^n (n=2,3) spectra obtained by LC-ion trap (IT) MS installing sonicspray ionization (SSI) source and by varying collision-induced dissociation (CID) voltage was tested paying attention to both fragment ions (m/z) and intensities. This is apparently essential for a development of reliable MS^n database aimes to the oligosaccharide structural assignment[5]. Then, a possibility of structural assignment of the isomers has been investigated by calculating correlation coefficients between their MS^n spectra. We present that the MS^n spectral matching based on correlation coefficients is useful and applicable to the structural assignment of relatively large divalent sodium-adduct Yn type fragmentations of PA derivatized fucosylated oligosaccharides.
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(69) **Automatic Online Detection, Assignment and Analysis of Carbohydrate Structures Contained in the Protein Data Bank**

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Protein glycosylation is probably the most common and complex type of post-translational modification encountered in proteins. Glycosylated proteins are implicated in a wide range of cell-cell and cell-matrix recognition events [1,2]. Oligosaccharides participate in the processes of cellular recognition which are important in infection, cancer, and immune response [3]. Many protein structures stored in the Protein Data Bank (PDB) [4] contain carbohydrates covalently attached in glycosylation sites and non-covalently bound as ligands. So far, only very few attempts to analyse special types of this carbohydrate related data were made [5,6]. Here, we present an approach to analyse the entire carbohydrate data contained in the pdb-files using automatic assignment routines to make this information easily accessible and searchable.
Our software PDB2LINUCS extracts the carbohydrate information from the pdb-files and describes them using the LINUCS-code [7], a linear, unique notation for carbohydrate structures. PDB2LINUCS detects carbohydrates on the basis of element types, atom connections and steric orientation. Among the more than 20.000 structures currently deposited in the PDB we were able to detect about 1.500 entries containing more than 5600 carbohydrate molecules. About 3000 of the carbohydrates were found in glycosylation sites (86% N-glycans, 14% O-glycans), the others are non-covalently bound ligands. Comparison of the detected carbohydrates with the residue information given in the pdb-files shows that there is a large list of errors in the database.
The identified carbohydrate related data are stored in the GLYCO-PROT extension of the SWEET-DB [8]. Since the assignment procedure works fully automatically, GLYCO-PROT can be easily updated with new entries deposited in PDB. The currently implemented analysis tools enable a user-definable recall of relevant geometric features (e.g. glycosidic torion angles, linkage between protein and sugar) as well as detailed analysis of the amino acids sequence composition around each glycosylation site. These features will open the possibility to perform a detailed analysis of the structural requirements of O- and N-glycosylation sites in glycoproteins to all interested scientists. The implemented automatic procedures guarantee, that such an evaluation can be done using always all available data.
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(70) **U-c Fingerprint: Glycoprotein Analysis Based on A Lectin Array**

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Glycoproteins are produced by cells as a mixture of glycoforms, in which the same protein scaffold is adorned with variable glycan structures. The specific glycoforms and their relative proportions vary significantly with host cell types and growth conditions.
Analysis of the glycan structure of a glycoprotein is challenging and recognition of the importance of glycan structure is rapidly increasing. The number of protein therapeutics under development is also increasing, yet production capacity for recombinant protein drugs has become rate limiting in meeting market need. Consequently there is significant effort to develop cell lines capable of producing large quantities of protein. These cell lines and the growth conditions optimized for protein quantity often alter the glycosylation patterns of the expressed glycoproteins. Therefore, obtaining clones and optimizing growth conditions which will retain desired glycosylation patterns is key to relieving the capacity shortfall for biologics manufacturing.
Procognia has developed an innovative technology for analyzing the glycan structures of intact glycoproteins. The analysis can be performed on crude samples in growth media, obviating the need for time-consuming purification and degradation steps. A sample volume of less than 200 µl with protein concentrations of <0.5 µM is sufficient to produce a quantitative analysis. The U-c fingerprint technology allows the analysis of a glycoprotein to be performed in hours by a technician with limited "hands on" time, and up to 40 samples can be run in parallel. With these features, the U-c fingerprint technology is applicable to all stages of clone selection and optimization, process development, growth condition monitoring, manufacturing and QC.
The method is based on an array of more than 30 lectins grouped with overlapping specificities. The glycoform mixture is bound to the array, and glycoform binding is detected by applying one or more labeled probes. The probes can be lectins or entities that recognize the protein moiety. The resulting fingerprint is characteristic of the glycan profile of the glycoform mixture, and is highly sensitive to changes in this profile. We have constructed a database of lectin-glycan recognition rules using a large dataset of carefully chosen, well-characterized glycoproteins. We can use this database and our proprietary algorithms to give quantitative analysis of glycan structures within 10% accuracy.

(71) **Development and Validation of DMB Assay in 96-well Microplate for Quantification of Glycosaminoglycans from Normal Urine and Urine from Patients with Different Types of Mucopolysaccharidoses**

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The Mucopolysaccharidoses (MPS) are a group of inherited lysosomal storage disorders of connective tissue. Many of these disorders are caused by a severe deficiency of an enzyme involved in the degradation of glycosaminoglycans (GAG). Excessive urinary excretion of GAG is commonly used as a screening test for all types of the MPS.
The quantification of GAG before and after treatment with enzyme is used to monitor the efficiency of MPS enzyme replacement therapy. Our assay is based on the dimethylmethylene blue (DMB) dye-binding spectrophotometric method of de Jong et al. (1) modified for 96-well microplates. Modification of this test tube based procedure for microplates enables the simultaneously processing of large numbers of samples in a short period of time. The method can be used for the determination of total GAG in biological fluids because different GAG species (heparan sulfate, dermatan sulfate, keratan sulfate, chondroitin-4- and -6-sulfates) have similar molar extinction coefficients. The method can be used for quantification of individual GAG species following

their degradation by different types of chondroitin lyases (eliminative cleavage) or/and by chondroitin hydrolases (hydrolytic cleavage).
The assay is linear from 1.25 µg/mL to 30 µg/mL of dermatan sulfate standard with an R^2 of 0.9998. There was no evidence for a urine matrix effect when several mixtures of normal and MPS II urine were tested. The assay was found reproducible, reliable and rugged with respect to analyst and day-to-day variables. The reportable values were found robust up to 30 minutes post assay termination when stored at room temperature and protected from light. Assay recovery in normal urine spiked with dermatan sulfate (up to nearly 3 mg/mL) was excellent. Assay specificity was demonstrated for polymeric GAG. A reproducible dose response was observed with increasing concentrations of dermatan sulfate, while chondroitin disaccharides produced negligible absorbance.
The effect of protein was examined. Human serum albumin was found to have a small effect on GAG quantification in normal urine but no effect on GAG recovery in MPS II urine. Hemoglobin, on the other hand, had no effect on the assay of normal urine, but appears to cause some interference at levels above 1.0 mg/mL in MPS II urine. Results are reproducible following treatment of urine to at least three freeze-thaw cycle, and storage up to 4 hours at room temperature.

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(72) *In Vivo* Production of N-Glycans Carrying LacdiNAc GalNAcβ1→4GlcNAcβ→R and PolyLacdiNAc Sequences

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Glycoproteins from a wide range of organisms have N-glycans containing GalNAcβ1→4GlcNAcβ1→R (LacdiNAc or LDN)-based structures. These include unmodified; 4-O-sulfated; α2,6-sialylated; and α1,3-fucosylated LDN. Although some of these structures have been synthesized *in vitro*, there is so far no convenient biological system available to produce glycoproteins carrying these structures on their N-glycans. To address this problem, we have generated a cell-line, derived from CHO-Lec8 cells, that expresses a cDNA encoding the *C. elegans* β1→4GalNAc-Transferase which we had previously characterized. This cell-line (L8-GT) produces N-glycans with LDN. Surprisingly, we also find novel polyLDN-type glycans with repeating LDN motifs (3GalNAcβ1→4GlcNAcβ1→). Glycans were characterized by MALDI-TOF analysis. In addition, we are currently generating additional cell-lines capable of synthesizing 4-O-sulfated; α2,6-sialylated; and α1,3-fucosylated LDN structures by transfecting L8-GT cells with cDNAs encoding several of the corresponding modifying enzymes. Characterization of the N-glycans produced by these cell-lines will be presented.

(73) Structures of *N*-Glycan from Human Dermis

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Oligosaccharides of lipids and proteins control cell developments and function as ligands for intercellular adhesion receptors, cell matrix proteins, growth factors, toxins and microbes. Many histochemical studies using lectins and antibodies recognizing saccharide moieties have shown the presence of various glycoconjugates in skin. However, detail structures of oligosaccharides, their ratios, and functions have not been clarified yet. In the previous studies, we showed that the most predominant *N*-glycan from murine dermis contain biantennary complex type oligosaccharide. Our attention has been directed toward structures of *N*-glycans carbohydrates displayed on human skin cells.
In this study, we analyzed and discussed detail structures and ratios of *N*-glycans from human dermis in comparison with murine dermis. Dermis was digested using trypsin and chymotrypsin. *N*-glycans were released from glycopeptides by using *N*-glycosidase F. The reducing ends of the oligosaccharides were labelled with 2-aminopyridine. The pyridylaminated oligosaccharides were subjected to the analysis by two-dimensional HPLC mapping technique.
Finaly, we determined 17 kinds of oligosaccharides of human dermis. It was suggested that complex type oligosaccharides were estimated to be 89.7% and high mannose type and hybrid type were 6.0% and 4.3%, respectively. On the other hand, murine dermis had 14 kinds of oligosaccharides. Complex type oligosaccharides were 86.2% and high mannose type and hybrid type were 11.8% and 2.0%, respectively. The major structure both from human and mice was Gal*f*À1-4GlcNAc*f*À1-2Man*f*¿1-3(Gal*f*À1-4GlcNAc*f*À1-2Man*f*¿1-6)Man*f*À1-4GlcNAc*f*À1-4(Fuc*f*¿1-6)GlcNAc. As a result, *N*-glycans from human dermis were quite similar to those from murine dermis.

(74) Glyco-Search-MS: a Web-based Tool to Support the Rapid Identification of N- and O-Glycans in MS spectra.

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The human genome seems to encode for not more than 30,000 to 40,000 proteins. This relatively small number of human genes compared with the genome of other species has been one of the big surprises coming out of the analysis of the human genome project. To better understand the functions of proteins, it is essential to grasp the features of their post-translational glycosylation, especially of N- and O-glycans. Although the same glycosylation machinery is available to all proteins that enter the secretory pathway in a given cell, most glycoproteins emerge with characteristic glycosylation pattern and heterogeneous populations of glycans at each glycosylation site. Therefore, glycosylation pattern are sensitive markers of changes within the environment of cells.
In recent years, rapid, high-sensitive MS strategies for screening glycans have been educed. However, there is still a need to develop methods for a reliable, quick and automatic evaluation of MS spectra to identify the N- or O-glycan repertoire. In analogy to other field of biochemistry where databases with reference spectra of suitably pure and homogeneous compounds are available, it can be assumed that appropriate intelligent database search algorithm will also applicable for an automatic identification of N- and O-glycans. Here, we describe the generation of databases containing all theoretically possible fragments of about 5000 N- and 1200 O-glycans. The glycan structures were extracted from SWEET-DB (1). The GLYCO-FRAGMENT algorithm (2) was used to calculate and assign the peaks (all A-, B-, C-, X-, Y-, and Z-fragments were generated) according the definitions of Domon and Costello (3)
An efficient search algorithm comparing each peak of a measured MS-spectrum with the calculated fragments of all entries contained in the database was implemented. The number of matched peaks within a certain tolerance is used to compute a score by which the best matching spectra are ranked. The search algorithm has been intensively tested for N- and O-glycans on the basis of MS spectra taken from the literature. The web-based Glyco-Search-MS interface allows an interactive interpretation of one spectrum at a time. The user has the opportunity to select the most common derivatization of the reducing sugar, like 2-aminopyridin or 2-benzamid. Additionally, the user has to tell if the experimental spectrum derives from permethylated or peracetylated glycans and which ESI ion has been used.
For series of MS spectra Glyco-Search-MS can be used in batch mode. A list of best matching spectra will be produced for each input list of experimental peaks. Each search takes less than a second on a standard PC. Thus, this approach is well suited for the rapid identification of known N- and O-Glycans in high-throughput projects
The service is available at: http://www.glycosciences.de/

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(75) Generating a Library of Oligosaccharides to Develop Sugar-Display Technology and Assess the Specificity of Carbohydrate Binding Proteins

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In this study, we introduce strategies to build up diverse series of glycans in a large-scale (up to 10 grams), which will be used in developing a library of carbohydrate compounds. This work demonstrates that chemoenzymatic synthesis of complicated carbohydrates can reach a facile and practical level

by employing a functional toolbox of glycosyltransferases. We have produced 24 different glycosyltransferases in bacterial, fungus, insect cell, and mammalian expression systems. Utilizing these enzymes has enabled us to synthesize various glycans such as; sialoside derivatives of N- and O linked, ganglioside mimics, blood group antigens, poly-N-acetyllactosamine and its corresponding fucosylated and/or sialylated compounds. We have chemically derivatized our compounds with a built-in neutral spacer that makes them suitable for practical manipulations such as matrix immobilization, biotinylation or polyacrylamide substitutions. The library of oligosaccharides, with a window of more than 80 different structures, has been established in our laboratory [1] and is currently being expanding. The versatility together with diversity makes this library favorable of being used in the newly developed sugar-display technologies in high throughput studies of carbohydrate-protein, as well as, carbohydrate-carbohydrate interactions. These methodologies can further be employed in constructing non-natural analog molecules with therapeutic values or entitled to drug discovery (an example of this type of application is presented in an independent abstract by our group). This library is currently in use for developing a glycanarray to conduct the specificity studies of carbohydrate binding proteins within the Consortium of Functional Glycomics. Both the glycanarray and the library of compounds will be made available through the Consortium for investigators both inside and outside the program. [1]
http://web.mit.edu/glycomics/consortium/resources/resourcecored.shtml

(76) Separation of Protein Glycoforms with Affinity Capillary Electrophoresis

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A growing interest in the significance of microheterogeneity in glycosylation of proteins has generated a need for improved analytical tools. It is desirable to analyse the intact glycoprotein rather than the free carbohydrates originating from the glycoprotein. The large number of similar glycoforms of an individual glycoprotein makes this a difficult task. Lectins can recognise specific carbohydrate groups and are often used for glycosylation studies. In this work we demonstrate the potential of using the lectin ConA as a pseudo-stationary phase in affinity capillary electrophoresis for analytical separations of protein glycoforms of α_1-acid glycoprotein (AGP, Orosomucoid) with a "partial filling technique". ConA separated AGP into two peaks with approximately the same size. The first peak includes AGP glycoforms that not interacts with ConA and the second peak include the AGP glycoforms that ConA recognizes. With the addition of different monosaccharides to ConA the specificity of the interaction could be studied.

(77) Capillary LC-Q-TOF Mass Spectrometry in Detection and Structural Analysis Of Glycopeptides in Complex Peptide Mixtures: Application to Glycosylation Analysis of Immunoglobulin G

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Recent advances in mass spectrometry and related hyphenated techniques, such as LC-MS and CE-MS, have made simultaneous detection and identification of glycopeptides in peptide mixtures mostly a routine analytical procedure (1). However, challenges still remain, especially in detailed glycosylation analyses of proteins belonging to the immunoglobulin superfamily. Inherent variable regions make enzymatic digests of these proteins extremely complex, containing large numbers of low abundant peptides whose glycosylation status has to be assessed. Biological significance of these molecules, as well as recent reports that they may contain unusual types of glycosylation (2), have made development of new approaches to study their glycosylation mandatory. Here we present a systematic approach for detection and identification of glycopeptides in enzymatic digest of the immunoglobulin G (IgG) molecule, using capillary LC coupled to a Q-TOF mass spectrometer. IgG presents a good model for evaluation of new analytical techniques, since it contains well described conserved N-glycosylation which can be used as internal control. IgG purified from human serum was digested with trypsin, reduced, alkylated, and resulting digest was pre-fractionated using a microbore RP HPLC coupled to a 3Q mass spectrometer (PE-Sciex, Concord, Canada). Fractions were collected and further analyzed on a capillary RP HPLC (LC Packings, Amsterdam, The Netherlands), coupled to a nano-ESI Q-TOF mass spectrometer (Micromass, Manchester, UK). Each fraction was run in triplicate: 1) as a standard LC-MS run; 2) as a LC-MS run with moderately increased collision gas pressure and collision energy to induce gas-phase deglycosylation; and 3) as a LC-MS run with highly increased collision gas pressure and collision energy, combined with automated MS-to-MS/MS switching to identify detected glycopeptides. In this approach, deliberately induced gas-phase deglycosylation has produced reporter sugar ions that revealed exact elution times of glycopeptides, whose identity was subsequently confirmed in the MS/MS run. IgG N-glycopeptides containing conserved N-glycans have been successfully detected, and their full structural characterization was possible. Reduction of complexity, i.e. prefractionation, was shown to be crucial for glycopeptide detection. These results demonstrate that capillary LC coupled to a Q-TOF MS is a sensitive and powerful tool in structural analysis of glycopeptides directly form extremely complex peptide mixtures. The data is currently being further investigated for presence of other proposed types of glycosylation on the IgG molecule, especially O-fucosylation. (1) Haynes PA and Aebersold R. 2000. Anal. Chem. 72: 5402-5410 (2) Gornik I, Floegel M, Peter-Katalinic J, Lauc G. Glycobiology 2001, San Francisco, CA, USA

(78) New Approaches for Analyzing the Bioactive Structures of Heparan Sulfate with Miniaturized LC/MS

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Heparan Sulfate polysaccharides interact with numerous proteins at the cell surface and orchestrate a myriad of biological events. Unraveling the mechanisms of these events at the molecular level require structural analysis of these negatively charged, heterogeneous biopolymers. Since these biopolymers are often available only in a small quantity, it is necessary to develop ultra sensitive methods such as mass spectrometry, which requires that samples be homogeneous, and be free from interfering alkali metal ions. A new strategy was developed using miniaturized LC/MS to analyze these biopolymers at disaccharide or oligosaccharide level. Analysis of Heparan Sulfate disaccharides harvested from wild type, 3-OST1, 3-OST3a and 3-OST5 sulfotransferase transduced Chinese Hamster Ovary (CHO) cells were carried out. Finally, the spacing of the rare GlcNAc-GluA disaccharide sequence, the acceptor site for 3OST1 action or cleavage site for endoglucouronidase action in the completely desulfated N-resulfated porcine intestinal heparin was determined. This region corresponds to the 3-OST1 precursor site and also corresponds to the partial structure of the ATIII-binding site. This disaccharide sequence occurs only at the distance of hexasaccharide, octasaccharide, decasaccharide or dodecasaccharide. Thus the current approach would allow one to decipher structure-function relationships of this special class of biopolymers. Despite the compelling evidences available for the role of HS in numerous biological events, only a handful of structure-function relationships have been characterized. The major theoretical problem is the heterogeneity in the sequence of the HS. To overcome this problem one is forced to identify critical functional groups within these heterogeneous polymers and determine the distance between the critical groups in order to establish structure-function relationships.

(79) Identification and Sequencing of Protein Binding Heparin-Like Glycosaminoglycan Oligosaccharides

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The heparin-like glycosaminoglycans (HLGAGs, heparin and heparan sulfate) are expressed on the surfaces of nearly all animal cells, in basement membranes and extracellular matrices. They consist of linear repeating units of GlcNAc(1-4)GlcA(1-4), variations in the arrangement of sulfated residues and uronic acid epimers of which comprise ligand-specific binding sequences. Because these sequences exhibit varying specificity for a number of HLGAG-binding proteins, the ability to perform mass spectrometric sequencing provides insight into mechanistic and regulatory studies.
The labile nature of these molecules provides an extremely challenging analytical problem. This work is an extended study to provide a full strategy facilitating the identification and isolation of HLGAG epitopes which participate in protein binding. This strategy also provides methods of obtaining maximal structural information of identified epitopes.

HLGAGs can be analyzed using negative ionization nanospray mass spectrometry. This coupled with an ion trap can facilitate collision induced dissociation studies of formed precursor ions. The extent of deprotonation present in the HLGAG precursor ion greatly influences the gas phase dissociation of the precursor ion and thus the nature of formed precursor ions. Precursor ions possessing a low degree of deprotonation experience facile losses of SO_3 and few cleavages at glycosidic bonds. Highly deprotonated precursor ions yield abundant product ions resulting from glycosidic bond cleavage thereby providing sequence information. However, the formation of high charge states is limited by like-charge repulsions. Some charge state control is provided through alteration of pre-ionization solvent conditions. Although tandem MS of solvent mediated deprotonated HLGAG ions provides a greater amount of sequence information, precursor ions may undergo losses of HSO_4^- to alleviate like-charge repulsions within the ion. Tandem MS of HLGAG ions formed by metal cationization provides deprotonation, thus retention of sulfate groups, without producing additional negatively charged sites. Through this method of analysis, abundant sequence information can be acquired without ambiguity as to the localization of sulfate groups. Also extensively studied are HLGAG ion behavior as influenced by various solvents and alkaline earth and transition metal binding to HLGAGs and the value of each with regard to (1) ion formation and (2) ion dissociation.

To target biologically relevant HLGAG sequences for tandem analysis, HLGAG mixtures are identified using an affinity size exclusion chromatography system. Using this system, HLGAGs binding proteins of interest are observed at a low elution volume. Detection using mass spectrometry allows the structures of these protein binding HLGAG sequences to be observed.

(80) **Glycan Disassembly by MS^n: Linkage, Branching and Monomer Identification**

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Molecular glycosylation and specific carbohydrate epitopes have emerged as important structural components for mediating biological function. A complete structural characterization of these entities is a challenging analytical problem that requires an understanding of isobars, inter-residue linkage, anomeric configuration, and branching. Analytical techniques such as Nuclear Magnetic Resonance (NMR) have been successfully applied for the characterization oligosaccharides, but these techniques still require relatively large amounts, as well as, pure oligosaccharides. Mass Spectrometry (MS) is the most effective analytical tool available because of its high sensitivity and lower sample consumption. The full extent of structural detail, however, remains largely unexplored. Sequential disassembly, MS^n, offers the best opportunity to identify structural features not observed in a single dimension spectrum. The disassembly to smaller residues is matched by an increasing number of specific fragments yielding greater structural understanding. By following selective pathways of disassembly, product fragments can be generated from large oligosaccharides that expose the difficult features of isomeric structure, linkage, and branching.

For example, collision induced dissociation (CID) of disaccharides of varying linkages, (1-2, 1-3, 1-4, 1-6), generated from MS^2 of their corresponding trisaccharides, indicates a clear and reproducible difference in their MS^3 spectra. Similarly, disaccharide fragments composed of similar monomers but differing only in the anomeric configuration (α, β) are identified, as are differences only in monomers, (Gal, Glc, Man). These spectral differences can frequently be rationalized on simple organic chemical principles. Importantly, these spectral differences were observed only from precursor ions generated *in situ*, strongly suggesting the gas-phase precursor ions to possess a 1,2 dehydro reducing terminus. Identical structures and their spectra have also been compared when generated *in situ* from different parent ions following different MS^n pathways, an indication the products are isoenergetic. This observation was fundamental for library building. Information obtained from the MS^n spectra of known glycans are now being used to assist with structure determination of unknown glycans obtained from of *C. elegans* (Andy Hanneman, adjacent poster) and to aid in the construction of bioinformatics tool. An MS^n library generated from standard glycans is being developed and used in conjunction with a composition library ("Composition Finder" Hailong Zhang, adjacent poster). Experiments were carried out using a Thermo-Finnigan LCQ and a Kratos MALDI-IT mass spectrometers. Supported by BRIN-NCRR(VR) and NIGMS(VR).

(81) **Composition to Sequence: A Novel Computational Approach to Support MS^n Carbohydrate Sequencing**

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Ion Trap mass spectrometry ($ITMS^n$) has been successfully applied to *de novo* sequencing of carbohydrate samples. This approach uniquely reveals structural details not observed in single dimensional experiments and provides an opportunity to develop bioinformatics tools. Here we report one computational approach in that regard. The strategy is built around a composition library of methylated carbohydrate structures and their MS^n fragments.

Fragments observed during MS^n disassembly provide "tell-tale" features of original structure, and these we define as "scars". As an example, free hydroxyl groups, cross-ring fragments, and eliminations suggest linkage, isobaric, and anomeric detail. Thus, a fragment ion composition becomes a rich source of structural information where multiple steps of disassembly bring an accumulation of scars reflected in unique mass product contributing to greater understanding. Orthogonal approaches can support such conclusions with analogs, derivatives, and cold isotopes. Importantly, the end-product of disassembly must define a structure, and it's pathway defines continuity and structural topology. These two elements of disassembly provide a foundation to propose bioinformatics studies.

In order to test the feasibility of the above approach, we use previously characterized *N*-linked glycans from ovalbumin, and sets of isobaric *N*-linked glycans isolated from *C. elegans* as our test case. However, the bioinformatics principles are general and not limited with indications that adjustments in derivative mass and compilation library should provide identical results. Two lists of mass/composition pairs of all possible *N*-linked glycans (with up to 20 hexoses, 6 deoxyhexoses, 20 HexNAc) and fragments (with up to 5 scars) are calculated by using a Python program. For the ease of data manipulation, a composition database was compiled by using MySQL to save as pre-calculated lists. "Composition Finder", a web interface to the database, is developed in PHP to implement the sequencing strategy described above. A "Snow Shovel" algorithm, was designed to rule out the impossible compositions, and also applied when the parent-daughter relationship are given on the input peaks.

In all test cases, this approach was able to successfully interpret structures complete with branching and linkage details. The results in all cases agreed with manual processing. And, since the tools incorporate all possible compositions, it helps to prevent the missing of alternative spectra interpretations, which is a common error during the manual processing. The tools can also help to identify which peak is the most structural informative one in order to avoid "opening the unnecessary doors". A generalized protocol based on the approach shows great potential as a fully automated procedure for high throughput applications. Supported by BRIN-NCRR(VR) and NIGMS(VR).

(82) **Structural Diversity in the Xyloglucans from Higher Plants in the Subclass Asterideae**

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Xyloglucans are major components of the walls of growing and developing cell in higher plants. These polysaccharides bind spontaneously and avidly bind to the surface of cellulose microfibrils, forming a network in the cell wall that prevents the cell from bursting under osmotic stress. The controlled, orientated expansion of this network regulates the morphological changes that give mature cells their characteristic shape and size, ultimately affecting the morphology of whole tissues, organs and plants. Xyloglucan sidechains terminated in the H-antigen (α-L-Fuc*p*-(1,2)-β-D-Gal*p*) are found in xyloglucans from the cell walls of most vascular plant species, including gymnosperms, angiosperms, and even lower plants, such as ferns. Evolutionary conservation of this structural feature suggests that it is a key factor that allows xyloglucan to perform its biological function in the cell wall. However, some groups of plants, notably those from the family Solanaceae (in the subclass Asteridae), lack this structural feature, replacing it with an α-L-Ara*f* residue. Furthermore, mutation or removal of the fucosyl-transferase gene responsible for the biosynthesis of the H-antigen in xyloglucans has no obvious phenotype in *A. thaliana*, and so the selective advantage provided by this structure must not come into play under greenhouse conditions. Xyloglucans from several Asterid species were analyzed to shed light on the evolutionary pattern that led to xyloglucan structural diversity in this subclass, revealing xyloglucans that contain a broad range of structural features,

including xyloglucans that appear to bridge evolutionary gaps between Solanceous plants and most other plant species.

(83) **Specific, Non-Reductive *O*-Linked Deglycosylation Coupled with Enhanced MALDI-MS Detection**

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A volatile organic base and a hemi-acetal capturing derivative have been combined to specifically release and trap *O*-linked glycans from glycoproteins, avoiding the need for irreversible *in situ* hydride reduction. The E_2 elimination from serine and threonine moieties proceeds smoothly and quantitatively with complete stability of *N*-linked glycans and phosphate esters. The common feature of base degradation, (peeling), has been avoided by trapping the released reducing terminus *in situ* with a quaternary amine, 2 aminoethyltrimethylamine, (AETMA). This covalently linked positively charge analog imparts *O*-glycans with enhanced sensitivity for MALDI-MS profiling, is easily released by mild hydrolysis, and stable to methylation. Collisional activation analysis, MS^2, indicates the AETMA moiety is readily lost providing a major product ion for detailed disassembly, MS^n. Other volatile amines have been found to protect hemi-acetals from base degradation. These observations have been tested with several standard structures, and applied to numerous *N*-, and *O*-linked glycoproteins. The described methodology suggests a number of advantages for the detailed structural characterization of glycoproteins.

Experiments were carried out using a Thermo LCQ and a Kratos MALDI-IT mass spectrometers. Supported by BRIN-NCRR(VR) and NIGMS(VR).

(84) **Characterization of Glycosylinositol Phosphorylceramides (GIPCs) and Glucosylceramide (GlcCer) from the Lectin-Producing Mushroom, *Polyporus squamosus***

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The polypore mushroom *Polyoporus squamosus* is the source of a lectin that exhibits a general affinity for terminal β-galactosides, but appears to have an extended carbohydrate-binding site with high affinity and strict specificity for the non-reducing terminal trisaccharide sequence NeuAcα2-6Galβ1-4Glc/GlcNAc [1]. Negligible binding was observed with oligosaccharides bearing the isomeric terminal trisaccharides NeuAcα2-3Galβ1-4Glc/GlcNAc or Galβ1-3(NeuAcα2-6)GlcNAc [2]. The specificity of the lectin towards NeuAcα2-6Galβ1-terminated glycans suggests considerable potential for its use in glycobiological studies, but its in vivo function is unknown. In considering the possibility that its function could involve interaction with an endogenous glycoconjugate, it would clearly be helpful to identify candidate ligands among various classes of carbohydrate-containing materials expressed by *P. squamosus*. Since evidence has been accumulating that glycosphingolipids (GSLs) may serve as key ligands for some endogenous lectins in animal species [3], possible similar roles for fungal GSLs could be considered. Two sub-classes of GSLs appear to be widely expressed in the fungal kingdom: (i) monohexosylceramides, such as glucosylceramide (GlcCer) and galactosylceramide (GalCer); and (ii) glycosylinositol phosphorylceramides (GIPCs), which comprise a structurally diverse class of anionic fungal (and plant) biosynthetic products, capable of bearing non-reducing terminal Galβ1- residues [4,5]. For this study, total lipids were extracted from mature fruiting body of *P. squamosus*. Multi-step fractionation, including DEAE-Sephadex anion exchange chromatography and preparative-scale HPLC, yielded a major monohexosylceramide component and three major GIPCs from the neutral and acidic lipids, respectively. These were characterized by a variety of techniques as required, including 1-D 1H- and 13C-NMR, 2-D 1H-1H-NMR, and 2-D 13C-1H-NMR spectroscopy; electrospray ionization mass spectrometry (ESI-MS and tandem -MS/CID-MS); monosaccharide, fatty-N-acyl, and sphingoid component analysis by GC/MS; and methylation linkage analysis by GC/MS. The monohexosylceramide component was determined to be GlcCer having a typical ceramide consisting of 2-hydroxy fatty-N-acylated (4E,8E)-9-methyl-sphinga-4,8-dienine. The two simplest GIPCs were identified as Manα1-2Ins1-P-1Cer and Galβ1-6Manα1-2Ins1-P-1Cer, respectively (where Ins = myo-inositol, P = phosphodiester, and Cer = ceramide consisting mainly of long chain 2-hydroxy and 2,3-dihydroxy fatty-N-acylated 4-hydroxy-sphinganines). The third major component is a complex pentaglycosyl-IPC consisting of a Galβ1-6Manα1-2Ins1-P-1Cer core glycosylated with three additional monosaccharide residues, one each of α-Fuc, α-Gal, and α-Man. The precise sequence is under investigation. Of these fungal GSLs, Galβ1-6Manα1-2Ins1-P-1Cer could potentially interact with *P. squamosus* lectin, and further investigations will focus on determining the binding affinity, if any, of the lectin for the GIPCs isolated from this fungus. [1] Mo H, Winter HC, Goldstein IJ (2000) J Biol Chem 275:10623-10629; [2] Zhang B, Palcic MM, Mo H, Goldstein IJ, Hindsgaul O (2001) Glycobiology 11:141-147; [3] Vyas AA, Patel HV, Fromholt SE, Heffer-Lauc M, Vyas KA, Dang J, Schachner M, Schnaar RL (2002) Proc Natl Acad Sci USA 99:8412-8417; [4] Barr K, Laine RA, Lester RL (1984) Biochemistry 23:5589-5596; [5] Jennemann R, Geyer R, Sandhoff R, Gschwind RM, Levery SB, Grone H-J, Wiegandt H (2001) Eur J Biochem 268:1190-1205.

(85) **Recombinant Expression of a *Leishmania* Side Chain Arabinose *(SCA1)* Gene in sf9 Insect Cells**

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Leishmania parasites utilize several strategies to survive and develop in the sand fly midgut, many involving the major parasite surface molecule lipophosphoglycan (LPG). Modifications of the basic LPG phosphoglycan (Gal-Man-P) backbone by side chain sugars play important roles in parasite survival in the sand fly and in vector competency. One of these LPG modifications requires the attachment of arabinose caps to side chain poly-Gals which facilitate the detachment of *L. major* parasites from the fly's midgut. We had previously identified two *SCA* (Side Chain Arabinose) genes involved in Ara attachment. *SCA1/2* exhibit canonical glycosyltransferase motifs and their overexpression leads to elevated microsomal β-AraT activity. Heterologous expression was used to determine whether the *SCA1* gene encodes the actual β-AraT. *SCA1* was cloned into the pFASTBAC1 expression vector with a poly-histidine epitope tag at its C-terminus and expressed in sf9 insect cells. The β-AraT activity of the recombinant protein was confirmed using microsomes from recombinant sf9 cells incubated with GDP-(3H)-Ara and purified LPG from *L. major* V1 (whose side chains terminate in bGal units) as an exogenous acceptor. Significant microsomal β-AraT activity (100 pmol Ara transferred/hr/mg protein at 30 C) was observed when incubated in presence of the LPG acceptor while no activity was observed in the absence of LPG. Product characterization was performed by depolymerization of LPG with mild acid and both Dionex HPLC and GLYKO FACE analyses of the resultant (3H)-arabinosylated fragments. The major peaks were identified as Ara-Gal-(Gal-Man) and Ara-Gal2-(Gal-Man). These results indicate that the *SCA1* encodes the β-AraT. Thus, the SCA1 protein defines a new family member of eukaryotic glycosyltransferases, and represents the first developmentally-regulated LPG modifying activity identified in *Leishmania*.

(86) **Type-IV pilin of Dichelobacter nodosus is Glycosylated**

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Dichelobacter nodosus, an obligately anaerobic gram negative bacterium, is the primary causative agent of ovine footrot. It produces polar type-VI fimbriae (pili) which are assembled from a protein subunit called pilin. Pilin plays a major role in both pathogenicity and the immunological response of the host. Type-IV fimbriae are found on a wide range of bacteria within the beta and gama subdivisions of the Proteobacteria (Dalrymple and Mattick, 1987). The type-IV fimbriae of Neisseria gonorrhoea and N. meningitidis (Power and Jenning, 2003) and Pseudomonas aeroginosa 1244 pilin (Comer, 2002) have been found to be glycosylated. Fimbriae appear to be the only proteins that are glycosylated on surface of Meningococci (Virgi et al., 1993). The literature suggests that fimbrial glycosylation may have direct functional consequences in pathogenesis (Virgi, 1997, Upreti, 2003). D. nodosus strains were isolated and identified from New Zealand and cultured in vitro. The fimbrial proteins were collected from New Zealand isolates and subject to denaturing sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). The calculated molecular weight of fimA from D. nodosus serotypes A, F does not correlate with their apparent size determined from electrophorectic mobility. For example, the pilin subunit of serogroup F which has the smallest calculated molecular weigh (15.800 Da), exhibits the largest apparent size

(20.000 Da) on SDS-PAGE. To confirm the discrepancy between calculated Mw and apperent Mr, isoated pilin of those strains firstly stained with periodic acid Schiff reagent (PAS) and deglycosylated by enzymatic glycosylation. These results suggest post translational glycosylation of the amino acid sequence in both outside and inside of the variable region of fimA.

(87) **Study and Construction of the Three N-Glycosylation Site- Specific Mutants of HCV E2 Glycoprotein**

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The hepatitis C virus genome encodes two membrane-associated envelope glycoproteins: E1 and E2. They are heavily modified by N-linked glycosylation. Recently it had been reported that the interactions between the glycosylation sites Asn-X-Thr/Ser and molecular ER chaperones are the key to the correct folding, assembling and maturation of viral proteins. Mutation of the fourth glycosylation site of HCV E1 protein was showed significantly enhancing the humoral response of both seroconversion rates and antibody titers. In order to study whether the mutagenesis of the HCV E2 glycosylation sites may affect the biosynthesis, assembly of viral proteins and the humoral immune response, we exploited the PCR method to introduce three glycosylation site-specific mutants into E2 protein. To construct three N-glycosylation site-specific mutants of HCV E2, pfx DNA polymerase were used and four synthetic oligonucleotide primers were designed. By the use of site-specific mutation method, two single and one double N-glycosylation mutants were obtained. In these N-glycosylation sites, Asn-X-Thr/Ser, Asn encoding codon was replaced by -Tyr- encoding codon. Two single site (A535T and A583T) mutants and one double sites (A535T, A583T) mutant of HCV E2 ligated in-frame into the vector pCDNA3.1 (-)/Myc-HisB were successfully constructed. The interactions between the molecular ER chaperones, calnexin, and the HCV E2 and its mutants had been studied by co-immunoprecipitation (IP) after they were being cotransfected into CHO cell line,. Different glycosylation mutants of E2 were used to immunize mice and their cellular and humoral immune levels had been checked through FCM. This work established the basis for further studies of processing of HCV structural proteins and the development of HCV new vaccine.

(88) **Urinary Tamm-Horsfall Glycoprotein from Various Species Differs in Its High-Mannose Structure and in How It Binds to Type 1 Fimbriated Escherichia coli**

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Tamm-Horsfall glycoprotein (THP) is the most abundant urinary protein in mammals (1). Previous studies have indicated that THP from human urine (hTHP) is implicated in the defense of urinary tract infections (UTI) in that it binds type 1 fimbriated Escherichia coli by its high-mannose glycan, and efficiently competes with cell-receptors exposed in the urinary tract, such as uroplakin-Ia and Ib, in adhering to these pathogens. In the present study, we observed some differences in THP glycomoiety isolated to the homogeneity from urine of various mammals. The major variations was detected in THP from pig urine (pTHP): (i) on sodiumdodecylsulfate gel pTHP exhibited faster mobility than hTHP, but this difference was annulled by removal of all N-glycans from the two THPs; (ii) Gala1,3Gal- epitope was found exclusively as the terminal non-reducing end of pTHP glycans while a1,3galactosyl-transferase, an enzyme absent from the human species, has been found to be active in the outer medulla of pig kidney; (iii) high-mannose structural analyses revealed that Man5GlcNAc2 occurred in pTHP in a much higher proportion than in hTHP (47% of total high-mannose sequences in pTHP versus 8% in hTHP); (iv) E. coli strains, bearing phenotypic variants on type 1 FimH adhesins prevalently occurring in UTI isolates, adhered to monomeric pTHP at a three fold higher level than was the case with hTHP, a result consistent with previous observations that Man5GlcNAc2 is the best inhibitor of type 1 E. coli-adhesion to cell receptors ; (v) pTHP showed a much higher resistance to leukocyte elastase hydrolysis than hTHP. Altogether these results support the notion that the host is able to produce protective agents against pathogens, under the selective pressure of a species-specific environmen. (1) Serafini-Cessi F., Malagolini N., and Cavallone D. Tamm-Horsfall glycoprotein: Biology and clinical relevance. Am. J. Kidney Dis. Vol. 42, October , 2003

(89) **Preparation of *Burkholderia pseudomallei* Polysaccharide – CRM_{197} Conjugate, a Potential Vaccine Candidate for Glanders and Melioidosis**

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Burkholderia mallei and *B. pseudomallei* are the causative biowarfare agents for glanders and melioidosis, respectively. They produce a capsular polysaccharide composed of 1,3 – linked homopolymer of 2 – *O- acetylated* 6-deoxy- β- D-manno-heptopyranosyl residues. A previous study of mice challenged with a *B. mallei* capsule mutant versus the wild type suggested that capsular polysaccharides were potential vaccine candidates for glanders. Currently, there is no protective antigen identified that could be developed as a candidate vaccine against glanders and melioidosis. Therefore, the main purpose of this investigation was to isolate capsular polysaccharide from *B. pseudomallei* in preparative quantities and then conjugate it to a carrier protein. We prepared a polysaccharide-protein conjugate, a potential vaccine candidate, for protection against glanders and melioidosis, in the following manner. A mixture of capsular polysaccharide and LPS was isolated from the cell pellet of *B. pseudomallei* 576 strain. The capsular polysaccharide and the *O*- antigen polysaccharides from LPS were released by acetolysis; they were separated from Lipid A and core oligosaccharides by size exclusion HPLC. The polysaccharide was conjugated to the diphtheria toxin mutant protein, CRM_{197} by reductive amination. Western blot analysis indicated that the antigen was indeed capsular polysaccharide and *O*-antigen derived from LPS.

(90) **Structural Characterization of Giant poly *N*-Acetyl Lactosamine from *T. brucei***

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African trypanosomes are extra cellular parasites of the mammalian vascular and interstitial system. The flagellar pocket; an invagination of the plasma membrane at the base of the flagellum, appears to be the only site for endocytosis and exocytosis of the parasites. The flagellar pocket presents electron dense 'matrix' material that binds ricin, tomato lectin and wheat germ agglutinin.

In order to understand the physiological role of the flagellar pocket, we started to study the chemical nature of the glycans which reside in this organelle. Osmotic lysed bloodstream parasites were solubilised in urea and SDS and glycoproteins were purified using ricin-coupled agarose. N-glycans were released by PNGase F digestion, purified by Bio-Gel P4 chromatography and further partitioned by high performance anion exchange chromatography (HPAEC).

The fine structure of ricin binding *N*-glycan was revealed by a combined approach including GC-MS, 1-D and 2-D, NMR, Qtof-MS and methylation linkage analysis. The ricin binding *N*-glycans present a complex biantennary structure, made of poly-*N*-acetyllactosamine. The poly-*N*-acetyllactosamine have a unique structure showing up to 40 (Galβ1,4GlcNAcβ1,6) repeating units with a β1,3–branching point every six repeats. Only 2 to 3% galactose residues are 3-substituted. The fine structure suggests that enzymic machinery involved in the biosynthesis of these *N*-glycans is unusual and may therefore present a potential as drug target. Fluorescence microscopy using FITC-ricin and other lectins that bind poly-*N*-acetyllactosamine (tomato lectin and Datura lectin) suggests that these *N*-glycans are located in both the flagellar pocket and the endosomal/lysosomal system. The structure and function of these high molecular weight poly-*N*-acetyllactosamine will be discussed in relation to the physiological and gel like appearance of the flagellar pocket matrix.

(91) **A Database of Pathogens and Their Carbohydrate Binding Affinities**

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The need for a comprehensive database of the carbohydrate binding affinities of pathogen adhesins has become overwhelmingly apparent in the past 10 years with the acceleration of research into the nature of microbial pathogenesis on the molecular scale. It is known that the presence of certain sugars on host tissue cell surfaces is sometimes predictive of susceptibility to infection by bacteria bearing complementary fimbrial adhesins. A database of the carbohydrate receptor sequences of microbial lectins has been compiled through an exhaustive search of literature published over the past 30 years. With applications in the fields of glycobiology and microbiology, this resource can be used to survey the carbohydrate affinities of human and domestic animal pathogens that have been studied to date. The prevention of certain

diseases through oral consumption of glycoprotein bearing target glycans such as galabiose has recently been elucidated. It is our hope that this resource will facilitate similar studies and will be sustained as a living online reference through the submission of new entries as more is learned about the glycobiology of disease.

(92) **An AFM Imaging-Based Assay for Viral Binding: Heparan Sulfate and Adeno-Associated Virus**

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Virus infections rely on the virion binding to cell surface receptors. Understanding the mechanisms involved in this interaction is relevant in research fields such as gene therapy, drug development and delivery, and biosensors. Heparan sulfate (HS) is known to play a role in the infection cycle of at least 16 viruses including adeno-associated virus type 2 (AAV2). We have developed an assay system which utilizes photolithography, functionalized silicon surfaces, PDMS mini-wells and atomic force microscope (AFM) imaging to study the specific interactions between HS and AAV. Viruses are adsorbed on to HS functionalized surfaces and imaged with the AFM. Using our system, four AAV serotypes (AAV1, AAV2, AAV3, and AAV5) were analyzed and the results show that HS binds to AAV2 and AAV3 specifically. We have also determined the apparent binding constant (K_d) of HS and AAV2 to be 340 pM. Our assay system provides a clean and controlled environment for observing virus-HS binding including the flexibility to study other binding mechanisms by modifying the immobilized HS or the virus. In addition, because the mini-wells in the assay are 3 microliters in volume, HS and virus usage are minimized to less than a microgram.

(93) **Invasion of Mammary Epithelial Cells by Toxoplasma Gondii Depends on Cell Surface Heparan Sulfate.**

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Heparan sulfate is composed of repeating disaccharides of variably sulfated glucosamine and uronic acid residues. Normally, the chains are found covalently attached to cell surface and extracellular matrix proteoglycan core proteins. Heparan sulfates contain binding sites for coagulation factors, growth factors, and selectins and therefore play a critical in a myriad of processes from development to blood clotting. They are also ubiquitously expressed in almost every mammalian cell type making them an excellent candidate for interaction with cellular pathogens including viruses, bacteria, and protozoa. Recently, Toxoplasma gondii has been shown to utilize highly sulfated heparan sulfate for attachment to mammalian cells. To further examine this interaction, we have initiated a project that directly assays the role of highly sulfated heparan sulfate in invasion of epithelia in vitro and in vivo. Using Cre-loxP technology, we have generated tissue specific knockouts of the enzyme N-deacetylase N-sulfotransferase I (NDST1) in mouse mammary epithelia. Cell lines purified from these mice exhibit heparan sulfate chains with reduced sulfation of the sugar chains. Tachyzoite (RH) invasion of knockout mammary epithelial cells is reduced compared to wildtype cells supporting previously published data that Chinese hamster ovary mutants in NDST1 were less susceptible to invasion by RH. Addition of NDST1 cDNA to the knockout epithelia restored wildtype invasion. However addition of purified heparin had no effect on RH attachment and invasion in wildtype or knockout epithelia. These findings suggest that the attachment of the heparan sulfate chains to proteoglycan core proteins may be critical for the invasion process. Studies are underway to test whether mammary epithelia lacking NDST1 are resistant to invasion by tachyzoites in vivo.

(94) **Manila Clam Lectin(MCL) Induced by Protozoan Parasite, u>Perkinsus, Involved in Host Defence.**

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New lectin(MCL) from manila clam(Ruditapes philippinarum was purified and characterized. Hemagglutination inhibition assay revealed that it binds to N-acetyl-galactosamine containing mucin-type carbohydrate. It also require Ca++ for it's activity. MCL was synthesized in hemocyte as 75kDa precuser and secreted into hemolymph. Non-reducing SDS-PAGE showed that it is dimerized by covalent bond(138 kDa). However MCL found in body extracted or Mucus fluid consist of two poly peptides(30, 34kDa in reducing condition) Parasite infection induced the MCL synthesis. Immunohistochemistry and western blot analysis showed that purifed MCL bind to the surface of Perkinsus hypnospore. Frozen section of infected Manila clam showed the infilteration of macrophage in infected area and the lectin was localized in around Perkinsus. The hemolymph from Perkinsus-infected manila clam cause the induction of MCL synthesis from the non-infected hemocyte.

(95) **Up-Regulation of Fucosyltransferase 5 in Cells Infected with Different Herpesviruses**

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Recent data indicate that herpesviruses are able to interfere with selectin-dependent inflammatory responses by inducing the Lewis substance, Lewis x (Lex) and sialyl Lewis x as decoys. Thus, up-regulation of FUT3, FUT4 and ST3GalIV, enzymes involved in the synthesis of Lewis x, has been shown in CMV infected cells. In the present study herpes simplex virus type 1(HSV-1), HSV-2, and CMV infected cells were compared to uninfected cells as to expression of fucosyltransferase 5. The expression of mRNA was performed using Real-Time TaqMan-dependent quantitative PCR. The PCR-systems were optimised and adapted for sensitive and accurate detection and quantification of glycosyltransferase gene transcripts in a one-step reverse transcription PCR using 18S rRNA as a housekeeping gene. The results from the screening of HSV-1, HSV-2 and CMV infected cells show an up regulation in the levels of FUT5 in HSV-1, HSV-2 and CMV infected cells compared to uninfected cells. This is not likely due to the binding of the virus, but post-binding events. The up regulation of FUT5 may constitute a molecular mechanism for the changed levels of bioactive surface glycans in infected cells.

(96) **Production of N-Linked Glycoproteins in *E. coli***

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The N-linked protein glycosylation is the most abundant post-translation modification of secretory proteins in eukaryotes. A wide range of functions are attributed to glycan structures covalently linked to asparagine residues within the asparagine – X – serine/threonine consensus sequence (Asn-Xaa-Ser/Thr). The synthesis of N-linked glycoproteins in the endoplasmic reticulum (ER) follows a complex, but highly conserved pathway in all eukaryotic cells. The initial step of this pathway is the assembly of a branched oligosaccharide on the lipid carrier dolichylpyrophosphate at the membrane of the ER. In the central reaction of the process, this lipid-linked oligosaccharide is then transferred to selected asparagines residues of newly synthesized polypeptides in the lumen of the ER catalyzed by the oligosaccharyl transferase. It is an enzyme complex consisting of nine highly conserved membrane proteins. Further subsequent species- and cell-type remodelling of the glycan structure takes place in the Golgi compartment. Stt3p is the most highly conserved oligosaccharyl transferase subunit among different species suggesting a direct role in N-linked protein glycosylation. Stt3p homologs are also found in archaea where N-glycoproteins are produced. However the discovery of a putative STT3 homolog in the genome of the Gram-negative bacterium Campylobacter jejuni was surprising and suggested N-linked protein glycosylation in this bacterium. We showed that C. jejuni is able to produce N-linked glycoproteins and that PglB is directly involved in this process, most likely acting as an oligosaccharyl transferase. Moreover, it became evident that the N-linked protein glycosylation pathway of the bacterium shows significant similarities to the N-linked protein glycosylation pathway of eukaryotes. The oligosaccharide is assembled on a carrier lipid at the cytoplasmic side of the plasma membrane, translocated to the periplasmic space and transferred to selected proteins. We further demonstrated that the functional N-linked glycosylation pathway of C. jejuni could be transferred into Escherichia coli. Though structurally the bacterial N-glycan differs from its eukaryotic counterparts, the acceptor peptide Asn-Xaa-Ser/Thr, where Xaa can be any amino acid except Pro, is essential for glycosylation of the protein. The cloning of a universal N-linked glycosylation cassette in E. coli opens up the possibility to engineer permutations of recombinant glycan structures for research and industrial applications.

(97) **Protein Glycosylation in the Enteric Pathogen *Campylobacter jejuni***

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Campylobacter jejuni is the first bacterium to show a system of general protein glycosylation, in which a heptasaccharide is transferred to Asn residues in multiple proteins, within the same Asn-Xaa-Ser/Thr sequon as eukaryotic glycoproteins (1,2). Previously, glycosylation of bacterial proteins had only been found in specific proteins such as the pili of *Neisseria* species (3) and mainly with O-linked glycans. The *C. jejuni* glycoproteins described so far were predominantly annotated as periplasmic proteins. We have extended the characterization of the products of the glycosylation system by examining extracts prepared under more strenuous conditions, and also inner and outer membrane preparations. Additional glycosylated periplasmic proteins were identified, as well as several inner membrane proteins, notably SecB a component of the secretion system. In addition, the genes for the glycosylation system, ie the *pgl locus* (4), were shown by genomotyping experiments with a complete DNA micro-array for *C. jejuni* to be present in all the >70 strains tested. This conservation is in complete contrast to the highly variable loci for the three other *Campylobacter glycans*, ie the lipooligosaccharide, the polysaccharide and the flagellin O-linked glycosylation. In order to extend the survey to other *Campylobacter* species and strains, experiments were undertaken with HR-MAS NMR. This method allows detection in whole bacterial cells of several anomeric signals from the N-glycan sugars, despite the presence of stronger signals from the polysaccharide (5). Signals attributable to the N-glycan moiety were found in two strains of *C. coli* and in *C. fetus spp venerealis*. Taken together, these experiments show that N-linked protein glycosylation is a widespread phenomenon in campylobacter. 1. Young, N.M. et al., 2002, J. Biol. Chem. 277, 42530-42539, 2002 2. Wacker, M., et al., 2002, Science 298, 1790-1793 3. Stimson, E., et al., 1995, Mol. Microbiol. 17, 1201-1214 4. Szymanski, C.M. et al., 1999, Mol. Microbiol. 32, 1022-1030 5. Szymanski, C.M., et al., 2003, J. Biol. Chem. 278, 24509-24520

(98) **Evidence for Glycosylation on a DNA-Binding Protein of A Strain of *Salmonella enterica*.**

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All organisms living in aerobic atmosphere have powerful mechanisms to protect macromolecules from oxygen reactive specimens. Thus, microrganisms developed biomolecule-protecting systems in response to starvation and/or oxidative stress. DNA biocrystallisation with Dps (DNA-binding protein from starved cells) is one example among these systems. Dps is a protein produced in large amounts when bacterial cell faces harm. An 18 kDa protein was purified from the crude extract of *Salmonella enterica* Typhimurium and characterised for its jacalin-binding ability. N-terminal sequencing revealed 100% homology with the Dps of *S. enterica* Typhimurium. Furthermore, α-metil-galactose inhibited the binding of Dps to jacalin in enzyme-linked lectin assay, suggesting that the Carbohydrate Recognition Domain (CRD) is involved in this interaction. Chemical deglycosylation with TFMS reduced the molecular mass slightly from 18587 to 18138 Daltons (449 Daltons), indicating that a small oligossacharide branch must be associated with its molecule.Finally, results from GC-MS showed that mannose is one possible constituent of the Dps glycan. Even though jacalin is known as a galactoside-binding lectin, there are some pieces of evidence that it might bind mannose as well. We are now working on structural and functional characterisation of the Dps glycan(s).

(99) **The spectrum of Carbohydrate Epitopes of Human and Animal Milk Glycoproteins As A Basis for Humanization of Animal Milk Glycosylation**

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Humans, as well as other species, are continuously exposed to different pathogens. Today, the most common treatment for bacterial infections is the use of various antibiotics: a treatment which can be complicated by the occurrence of pathogenic strains resistant to commonly used antibiotics, as well as patient allergy to antibiotics. Host cell adhesion of pathogens is a prerequisite for most infections, and for many bacteria, viruses and bacterial toxins, binding is mediated by lectins that recognize and bind to different carbohydrate epitopes. Breast-milk has a well-known anti-microbial effect, one reason being the complex carbohydrate structures found on different glycoconjugates and as free oligosaccharides. However, the spectrum of carbohydrate epitopes in breast-milk differs between species, with human milk expressing the most complex one. In recent years, the technology for generation of transgenic animals has improved, and transgenic animals are now being used as bioreactors for production of recombinant proteins in their mammary gland. Thus, glycosyltransferases transgenically expressed in the breast gland can be used to modify existing milk glycoconjugates for production of specific, humanized carbohydrate epitopes. Such milk may have a therapeutic value in terms of prevention or treatment of microbial infections. In order to identify species with a milk carbohydrate phenotype which upon transgenic modification would allow the biosynthesis of carbohydrates of potential therapeutic value, we have investigated the expression of protein-bound carbohydrate epitopes in milk from man, cow, goat, sheep, pig, horse, dromedary and rabbit. The proteins were separated by SDS-PAGE and carbohydrate epitopes detected by Western blotting using antibodies and lectins of known carbohydrate specificity. The precursor chains of several carbohydrate epitopes mediating pathogen-binding was found in milk from all animals investigated, as where the binding epitopes themselves. For instance, the Lewis^b and sialyl Lewis^x epitopes, known to mediate adhesion of Helicobacter pylori to gastric mucosa, were seen on proteins from man and pig milk. The Lewis^b epitope is produced by the action of fucosyltransferase III (FUT III), an enzyme adding fucose in an α1,4 linkage to the GlcNAc residue in H type 1. The blood group antigen H type 1 was expressed on milk proteins from all animals investigated. Furthermore, NeuNAcα2,3LacNAc, a precursor for sialyl Lewis^x, was also expressed on milk proteins from all species investigated. Production of sialyl Lewis^x, from NeuNAcα2,3LacNAc, can be done by several different fucosyltransferases (FUT III, FUT V-VII). In conclusion, transgenic expression of above mentioned glycosyltransferases might result in animal milk with the capacity to inhibit H.pylori colonization.

(100) **Structures of the *O*-Linked Oligosaccharides of A Complex Glycoconjugate from *Pseudallescheria boydii***

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A complex glycoconjugate from the mycelia of the human pathogen Pseudallescheria boydii was immunologically distinct from another from Sporothrix schenckii. Both N- and O-linked glycan structures were present. Three O-linked oligosaccharide fractions were liberated via sodium borohydride-mediated alkaline b -elimination. Their structures were partially determined using H-1 and C-13 NMR spectroscopy, MALDI-TOF-MS and ESI-QTOF-MS, and methylation analysis. The most likely structure of the largest oligosaccharide was a branched hexasaccharide terminated by 2,6-di-O-substituted mannitol, with the O-2 substituent being a "main chain" of a -Rhap-(1® 3)- a -Rhap- (1® 3)- a -Manp-(1® while the "side chain" at O-6, was a -Glcp-(1® 4)- b -Galp-(1® . A pentasaccharide lacked the terminal Rhap unit.

(101) **Glycosylation in *Schistosoma mansoni* Secreted Glycoproteins**

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Schistosomes are parasitic trematodes that cause schistosomiasis, which infects an estimated 200 million people worldwide. One of the most striking features of schistosomes is their ability to survive for extended periods in an

immunocompetent host. Increasing evidence indicates that glycoconjugates in schistosomes are important determinants that interact with the host immune system. Thus, it is important to elucidate glycan structures that *S. mansoni* carries in order to facilitate further understanding of the molecular basis of host-parasite interactions. Previous structural studies on *S. mansoni* have been based on total glycoprotein extracts from the cercaria (infective to man) and egg (responsible for transmission to the snail intermediate host). Characteristic features found in both stages are: (i) the Lewis X epitope, (ii) multifucosylated terminal structures and (iii) core xylosylation in N-glycans. Yet, little is known about *S. mansoni* secretions. In this study, N- and O-glycan profiles from glycoproteins secreted at different stages of the *S. mansoni* lifecycle were compared using matrix assisted laser desorption ionisation (MALDI-MS) and electrospray collisionally activated decomposition (CAD ES-MS/MS) mass spectrometry. Glycans from secreted glycoproteins were released using successive PNGase F and PNGase A digestions followed by reductive elimination. Released glycans were permethylated and analysed using MALDI-MS for rapid screening of the complex sample mixture and CAD ES-MS/MS to obtain detailed molecular structures of individual components. Results have revealed remarkable differences between the N- and O-glycan profiles from egg secreted proteins (ESP) and cercarial secretions (CS). In contrast to the CS, the ESP possess a larger repertoire of glycans and lower levels of N-glycan core xylosylation. Similarly, the ESP expresses a more diverse array of O-glycans than the CS. The secreted glycoproteins were underfucosylated compared to structures previously described in the cercarial glycocalyx O-glycans, egg glycosphingolipids and egg glycoprotein extracts. It is anticipated that these findings may lead to a better understanding of the roles and the significance of the difference in glycan repertoire plays in schistosomiasis.

(102) **Point Mutations in *embC* Affect Synthesis of Lipoarabinomannan in *Mycobacterium smegmatis***

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The cell wall of mycobacteria consists of a "core" which has been defined as a covalent complex of mycolic acid-arabinogalactan-peptidoglycan. Another major component in the cell wall is the non-covalently bound lipoglycan, lipoarabinomannan (LAM), a potent immunomodulator in tuberculosis and leprosy. The structure of the arabinan in arabinogalactan (AG) and LAM are not completely elucidated but both are composed of arabinose in the D-furanose configuration (D-Ara*f*), a very unusual sugar structure in nature with a xenobiotic status in humans. So far, only a few enzymes involved in the synthesis of these mycobacterial cell wall components have been identified, among them are the three Emb-proteins. These proteins are of interest, as their overexpression produces low levels of resistance to the drug ethambutol (a first line drug against tuberculosis) in an otherwise susceptible host (Belanger *et al.*, 1996). They are proposed to encode for mycobacterial arabinosyltransferases involved in AG (EmbA and EmbB) and LAM synthesis (EmbC) (Escuyer *et al.*, 2001; Zhang *et al.*, 2003). An amino-acid motif, with conserved proline residues, found in several bacterial membrane proteins has been shown to be involved in polysaccharide synthesis (Becker & Pühler, 1998). We have now identified this so called "proline-rich" motif in the EmbA, EmbB and EmbC proteins of several mycobacteria and their homologs are also present in corynebacteria. Zhang *et al.* (2003) recently reported that a knock-out mutant of the *embC* gene in *Mycobacterium smegmatis* generates a strain that is not capable of synthesizing LAM, but only lipomannan (LM). In our present work, we complement the *M. smegmatis* ΔembC strain with the plasmid pVV16 containing the embC gene mutated in the proline-rich motif by site-directed mutagenesis. Lipoglycans were extracted and the compositional analysis of LAM showed 50% less arabinose moieties than the wildtype according to sugar composition analysis by gas chromatography. Further, digestion of this altered LAM with an enzyme, endo-arabinanase, showed a profile distinct from the phenotype of LAM from *M. smegmatis*. Purified AG from complemented strains showed only marginal differences in the sugar composition of wild-type AG. In summary, point mutations in the proline-rich motif in EmbC affect LAM synthesis and produce a low molecular weight component, and do not affect AG synthesis per se, confirming earlier work that shows the arabinan of AG and LAM utilizes different sets of enzymes.

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(103) **Impact of Specific Neutral Human Milk Oligosaccharides on Gut Integrity in Egyptian Infants.**

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Development of mucosal enteropathy in infants in developing countries has been shown to begin during weaning when food is introduced that is likely to be contaminated (Lunn *et al.* 1991). Bacterial adherence to cultured intestinal epithelial cells has been shown to precede deterioration in permeability of the epithelial monolayer. Human milk oligosaccharides (HMO) may exert anti-adhesive properties by acting as soluble receptor ligands for bacteria invading the intestinal mucosa (Newburg, 1999). To test the hypothesis that HMO may protect the gut mucosa, intestinal permeability of 2–12-month-old Egyptian infants was measured from the urinary recovery of the orally-administered lactulose and mannitol. Both leakage in the paracellular pathway (increased lactulose passage) and villous atrophy (decreased mannitol recovery) will increase the lactulose:mannitol (L:M) ratio. L:M ratios of exclusively breast-fed and partially breast-fed infants were compared and related to the concentration of HMO and HMO fractions of their mothers. Infants who received only formula instead of breast milk served as controls. HMO were separated into acidic and seven neutral fractions by gel permeation chromatography and monitored by refractive index detection (Thurl *et al.* 1991). The presence of infections increases intestinal permeability. Urinary neopterin was used as a sensitive marker for sub-clinical infections. Most infants were from poor socio-economic backgrounds.

Intestinal permeability did not differ between exclusively and partially breast-fed children at any age but it was worse in older infants, i.e. urinary L:M ratios increased with age to abnormal values (>0.12) mainly due to decreased mannitol absorption. The highest L:M ratios were found in infants of the control group, which differed significantly from those in the breast milk consuming groups (exclusively and partially breast-fed infants combined) ($P<0.01$) at <4 months. When all age groups were combined the control group had higher L:M ratios ($P<0.05$) and lower mannitol:creatinine (M:C) ratios ($P<0.05$) than infants of breast milk consuming groups combined. Urinary neopterin concentrations were used to remove the age-associated influence of infection on L:M and M:C, to generate residuals.

Concentrations of total HMO and most neutral fractions declined through lactation. Regression analysis showed correlations between certain HMO fractions and gut integrity markers (both logged). About 20% (negative, $P<0.001$) of the variance in the L:M residuals was explained by both, the influence of a neutral fraction containing HMO (>1500 Da) and respiratory infection. This result remained even when the variable "age" was included into the analysis. The same HMO fraction also explained ~19% (positive, $P<<0.001$) of the variance in residual M:C. However, when age was included, only age explained the variance of residual M:C ratios (negative, $P<0.001$). The negative association between neutral HMO of higher molecular weight and the L:M permeability ratio support the hypothesis that certain HMO prevent a deterioration of intestinal integrity. The positive relationship of the same neutral HMO fraction with the M:C ratio shows that small bowel villi structure, i.e. absorptive capacity, seems to be preserved by this HMO fraction. However, correcting gut integrity markers using neopterin did not fully remove age-associated deterioration probably because of the accumulating effects of infection on the gut which is not fully removed by correction with neopterin, a marker of acute infection. In contrast although most HMO fractions decline with age, this represents a physiological change in milk composition and is not infection-related. The protective effects of certain HMO fractions against gut integrity needs to be confirmed in other populations groups as well as by intervention studies

Ethical consideration: The study protocol was approved by the Head of the Social and Preventive Pediatric Center, Cairo University Childrens' Hospital. Informed consent was obtained from the parent(s). Acknowledgements: The authors acknowledge the assistance from an International Training Fellowship (No. 2000-T1) of the Nutricia Research Foundation to carry out this work.

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(104) **Potent Glycomimetic Inhibitors of the Adhesion Molecule, PA-IIL, for the Bacterial Pathogen, *Pseudomonas aeruginosa*.**

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Pseudomonas aeruginosa is a ubiquitous pathogen that infects a wide variety of tissue sites in humans with compromised immune systems. In particular, this bacterium is the major cause of morbidity and mortality in patients with Cystic Fibrosis (CF). Infection and colonization of CF lungs follows a cyclical course of infection and inflammation eventually resulting in destruction of the lungs and death. CF is a genetic disease caused by mutations that alter chloride channels but also affects the glycosylation of lung tissue. Recent reports (Scanlin, T.F. and Glick, M.C., BBA 1455:241-253, 1999) demonstrate a net increase in fucosylation and decrease in sialylation. *P. aeruginosa* binds strongly to these modified carbohdyrates and colonizes CF lung tissue through the expression of surface lectins, PA-IL and PA-IIL (Gilboa-Garber, N., Methods Enzymol. 83:378-385, 1982). Blockade of these lectins presents a novel therapeutic strategy for *P. aeruginosa* infection. Here we report small molecular glycomimetic compounds that strongly bind and inhibit the PA-IIL lectin. IC_{50} values in the nanomolar range were determined by *in vitro* assays with purified lectin. These glycomimetic compounds retain the correct bioactive conformation and possess properties more compatible for drug development than native carbohydrates.

(105) **Identification of Genes Involved in the Biosynthesis of the Glycosyl Modifications on *Campylobacter jejuni* Flagellin**

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Campylobacter spp. are the leading cause of food-borne illness in the U.S. and are among the most frequently isolated causes of bacterial diarrhea in the world. Motility of these organisms is necessary for gastrointestinal colonization and for invasion of intestinal epithelial cells *in vitro*. Flagellin is the immunodominant antigen recognized during infection and is considered a major virulence factor. Flagellins from *C. jejuni* 81-176 are heavily glycosylated with pseudaminic acid residues and analogs. It has been shown that loss of the ability to glycosylate flagellin renders the organisms non-flagellated and non-motile. However, little is known regarding the enzymes involved in the biosynthesis of these glycosyl moieties. Identification of the biosynthetic genes will provide a better understanding into the role of glycosylation in flagella assembly and virulence. Using a combination of biochemical and mass spectral techniques, and microarray expression profiling, we have identified a spectrum of genes involved in the biosynthesis of the glycosyl modifications found on *C. jejuni* flagellin.

(106) **Pigeon Ovalbumin Glycans Labeled with Digoxin Can Be Used for Identification of Uropathogenic *E. Coli* (Type-P)**

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E. coli's are by far the most common cause of urinary tract infections. Standard methods for identification of uropathogenic *E. coli* (UPEC) in urine are semi-quantitative and usually take 2-3 days, so development of a more rapid, molecular approach to detect UPEC may lead to earlier diagnosis and improve management of urinary tract infections.
Attachment of UPEC to host cell receptors is mediated by interaction of the bacterial lectin (adhesin) localized at the top of fimbriae and specific carbohydrate moiety of the host membrane glycoconjugates. The most common type of UPEC causing pyelonephritis expresses type P adhesin (UPEC-P) that specifically recognizes P1 antigen (Galα(1-4)Gal moiety – galabiose). Pigeon glycoproteins are rich in galabiose sequences at the terminal positions of N-glycans that makes them potent ligands for UPEC-P adhesins. Oligosaccharides were purified from pigeon ovalbumin, reductively aminated with hydrazine and labeled with digoxin that was previously activated with CNBr.
Microtiter plates were coated with purified pigeon ovalbumin and incubated with solution containing bacteria or a model lectin. Pigeon ovalbumin glycans conjugated with digoxin were added and detected with antibodies against digoxigenin (that also recognizes digoxin) conjugated with alkaline phosphatase. Linear response was observed in a wide range of concentration, indicating that this method might be useful for quantifying bacteria. As far as we know this is the first case of labelling glycans with digoxin as well as their application.
Our preliminary study indicates that specific glycoproteins and glycans labeled with digoxin can be used for development of a rapid, species-specific molecular tool for detection of uropathogenic *E. coli*. Similar approach may be further utilized for the detection of other bacterial species, measurement of bacterial virulence and sensitivity to antibiotics.

(107) ***Mycobacterium tuberculosis Rv3782* Encodes A Galactofuranosyl Transferase Involved in Cell Wall Synthesis**

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Tuberculosis is increasing in prevalence worldwide, and, with the advent of multidrug-resistant tuberculosis, new drugs and drug targets are needed to halt the spread of the disease. The cell envelope of *M. tuberculosis* is essential for the growth and survival of this bacterium and steps involved in its formation represent potential drug targets for the treatment of tuberculosis. The cell envelope of mycobacteria is composed of the cell membrane, a cell wall region, and a capsular layer. Arabinogalactan is the primary polymer within the cell wall of *Mycobacterium spp.* This complex polymer is tethered onto *N*-glycolyl-muramic acid residues of the peptidoglycan layer by a linker unit composed of (-α-L-Rha*p*-(1→3)-α-D-GlcNAc-P). During formation of the polymer, the linker unit is attached to a polyprenol-P acceptor molecule, and synthesis proceeds through glycolipid intermediates. One of the research interests of our laboratory is the biosynthesis of the arabinogalactan polymer, specifically at the present time, galactan synthesis. Previously, we implicated Rv3808c in the formation of much of the galactan (J. Biol. Chem. 275; 2000). Bioinformatic analyses using PHI-BLAST, cluster analysis, and motif searches pointed to Rv3782 as a possible glycosyltransferase. Partial sequences of *Rv3808c* were applied in a BLAST search against the *M. tuberculosis* genome to identify potential galactosyltransferases, and *Rv3782* showed homology.
Galactosyltransferase assays were performed in reaction mixtures containing membrane and cell wall-enriched fractions from *M. smegmatis* mc^2 155 overexpressing *M. tuberculosis* Rv3782 in the presence of UDP-GlcNAc and TDP-Rha as precursors of the linker unit and UDP-[^{14}C-U]Gal as a precursor of galactan. Reaction mixtures were sequentially extracted with $CHCl_3$:CH_3OH (2:1) as a source of simple polyprenyl-P linked glycolipid precursors and more polar lipid solvents as a source of polyprenyl-P linked oligosaccharide precursors. The $CHCl_3$:CH_3OH (2:1) extracts were analyzed by thin-layer chromatography and autoradiography. More polar solvents were used to extract products with a higher degree of glycosylation and the extracted products were electrophoresed using gradient tricine gels, transferred to nitrocellulose, and visualized using autoradiography. The results show that the Rv3782 overexpressor, compared to the vector control, produced elevated levels of the simpler glycolipid precursors such as Gal-Rha-GlcNAc-P-P-polyprenyl and Gal_2-Rha-GlcNAc-P-P-polyprenyl as well as larger, more polymerized products. Thus, Rv3782 catalyzes the early and perhaps the initial galactosyltransferase step(s) in galactan formation.

(108) **Status of Sialoglycans in Indian Leishmaniasis**

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Sialic acids and their O acetylated derivatives are known to modify various biological phenomena. Exploiting the preferential binding of Achatinin-H towards 9-OAcSA linked to a subterminal GalNAc in an a2®6 linkage, the presence of this glycotope was identified on erythrocytes of patients with Visceral Leishmaniasis (1) and utilized for monitoring their disease status (2). Six molecular determinants were identified only on diseased erythrocytes that

disappeared following elimination of parasite burden (3). They were purified and utilized to develop an ELISA for monitoring the disease status (3). As the presence of 9-OAcSGs on mammalian erythrocytes enhances hemolysis via activation of the alternate pathway of complement (4), we observed a similar analogy on erythrocytes of VL patients that may contribute to the anemia associated with Visceral Leishmaniasis (3). As the topography of Leishmania parasites with regard to their sialoglycan profile remains a poorly investigated area, we have addressed this question in digenetic Leishmania donovani parasites. Both forms possess sialic acids and to a minor extent Neu5, 9Ac2 (5, 6). Their linkage specificity was confirmed using sialic acid binding lectins Sambucus nigra agglutinin, Maackia amurensis agglutinin and an O-acetyl sialic aid binding lectin, Achatinin-H. As no measurable UDP-GlcNAc 2-epimerase activity was evident on promastigotes, it indicated that Leishmania possibly do not have a sialic acid biosynthetic machinery; instead, they adsorb serum sialoglycans (5). Taken together, the study of sialoglycans especially with regard to host-parasite interaction in Leishmaniasis may be helpful in disease management (7). References: 1 Sharma V, Chatterjee M, Mandal C, Basu D, Sen S. Rapid diagnosis of visceral leishmaniasis using AchatininH, a 9-O-acetyl sialic acid binding lectin. Am J Trop Med Hyg 1998; 58:551- 54 2 Chava AnilKumar, Chatterjee M, Sundar S, Mandal C. Development of an assay for quantification of linkage-specific O-acetylated sialoglycans on erythrocytes; its application in Indian visceral leishmaniasis. J Immunol Methods 2002; 270:1-10. 3 Chava AnilKumar, Chatterjee M, Sundar S, Mandal C. Differential expression of O-acetylated sialoglycoconjugates induces a variable degree of complement-mediated hemolysis in Indian Leishmaniasis. J Inf Dis (under revision) 4 Sharma V, Chatterjee M, Sen G, Chava. Anil Kumar, Mandal C. Role of linkage specific 9-O-acetylated sialoglycoconjugates in activation of the alternate complement pathway in mammalian erythrocytes. Glycoconjugate J 2000; 7: 887-93. 5 Chatterjee, M., Chava, Anil Kumar, Kohla, G. et al., Identification and characterization of adsorbed serum sialoglycans on Leishmania donovani promastigotes. Glycobiology 2003, 13, 351-361 6 Chava Anil Kumar, Chatterjee M, Gerwig GJ, Kamerling JP and Mandal C. Identification of sialic acids on Leishmania donovani amastigotes. Biol Chem Journal (Under review). 7 Chava Anil Kumar, Bandyopadhyay S, Chatterjee M and Mandal C. Sialoglycans in protozoal diseases; their detection, modes of acquisition and emerging biological roles. (Invited Review) Glycoconjugate J (In press).

(109) Altered Nuclear Pore Glycosylation in *Caenorhabditis elegans* Lacking O-Linked-N-Acetylglucosaminyltransferase

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O-linked N-acetylglucosamine (O-GlcNAc) modifies nuclear pore proteins, kinases and transcription factors. Disruption of the mammalian O-GlcNAc Transferase (OGT) gene leads to stem cell and embryonic letality. Although essential in mammals, the precise role of O-GlcNAc is unclear. To examine the function of O-GlcNAc in a genetically amenable organism, we have identified a viable null allele of O-GlcNAc transferase (ogt-1(ok430)) in Caenorhabditis elegans. We demonstrate that while nuclear pore proteins of the homozygous deletion strain completely lack O-GlcNAc, nuclear transport of a number of transcription factors including SKN-1, MyoD, and daf-16 appears normal. In addition, the deletion strain appears to develop normally with little change in lipid storage, growth rate or fertility. We discuss these findings in terms of the proposed functions of O-GlcNAc transferase in nuclear transport, transcriptional repression and intracellular signaling.

(110) Insulin Resistance in Adipocytes as Microdomain Syndrome: Involvement of Ganglioside GM3

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Numerous recent studies have implicated the involvement of TNFa in insulin resistance in adipocytes in culture as well as in whole-animal models, but its mechanism of action is not fully elucidated. A uniform finding in insulin resistance has been shown that tyrosine phosphorylation of insulin receptor substrate-1 (IRS-I) in response to insulin is decreased in adipocytes and skeletal muscle in obesity and type 2 diabetes. Ganglioside is a family of sialic acid containing glycosphingolipids(GSLs) and adipose tissues from various species, including human, rabbit, rat, and mouse, contain GM3 as the most abundant type of ganglioside. We previously found that in the murine 3T3-L1 adipocytes aquired the chronic state of insulin resistance induced by prolonged treatment with relatively low concentration (100 pM) of TNFa, the synthesis of GM3 was upregulated, and the inhibition of GM3 synthesis by an inhibitor of glucosylceramide synthase (D-PDMP) normalized the insulin signaling from insulin receptor (IR) to IRS-I (Tagami S, Inokuchi J., Kabayama K., Yoshimura H., Uemura S., Orawa C., Ishii A., Saito M., Ohtsuka Y., Sakaue S., and Igarashi Y. J. Biol. Chem. 277, 3085-3092, 2002). We could extend these in vitro observations to intact animals, since GM3 synthase mRNA levels and its chemical contents in adipose tissues from the obese Zucker fa/fa rats and ob/ob mice were significantly high in comparison to their lean animals, suggesting the involvement of GM3 in the pathological conditions of insulin resistance in type 2 diabetes. These our findings are supported by the observation that knockout mouse lacking GM3 synthase exhibits the enhancement of insulin signaling (Yamashita Y. et al. Proc. Natl. Acad. Sci. 18, 3445-3449, 2003) Cellular membranes contains sub-domains termed lipid ordered domains or GSL signaling microdomains. These sub-domains, characterized by their detergent-insolubility, are highly enriched in cholesterol and GSLs, and de-enriched in phospholipids, therefore, we called here this specialized domain detergent resistant microdomains (DRMs). Since GSLs including GM3 is an important component in DRMs, we have performed several critical experiments to examine the role of increased GM3 in organization and functions of microdomains. Using TNFa treated 3T3-L1 adipocytes as a model of chronic state of insulin resistance, we found that 1) IR was selectively less accumulated in DRMs and shifted to the high density fractions. 2) Insulin-stimulated IR internalization from plasma membranes and the intracellular movement of IRS-I were greatly suppressed, leading to the uncoupling of IR-IRS-1 signaling. Insulin-dependent tyrosine phophorylation of IRS-I was selectively impaired without affecting the autophosphorylation of IR and the MAPK activation. 3) D-PDMP treatment was able to cancele the TNFa-induced inhibition of both insulin-stimulated IR internalization and elimination of IR from DRMs. 4) Direct involvement of GM3 was proved by transfection of GM3 synthase gene constructed with adenovirus vector into normal 3T3-L1 adipocytes, showing the selective inhibition of insulin-dependent IRS-1 tyrosine phosphorylation. Taken together, our current findings strongly suggest that the defect of insulin's metabolic signal in insulin resistance could be attributed to the loss of IR from DRMs due to the accumulation of GM3.

(111) Role of N-Linked Glycans of Ganglioside GM3 Synthase (SAT-I)

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Glycosphingolopids are components of eukaryotic plasma membrane, especially membrane microdomain. Ganglioside GM3, which contains a sialic acid, is one of the glycosphingolipids. We have reported that GM3 is involved in the insulin resistance of 3T3-L1 adipocytes, and that ganglioside GM3 synthase (SAT-I) gene in the model animals of type 2 diabetes, fatty mice, increase remarkably compared with their lean counter parts. Moreover, it has been reported that insulin sensitivity in the SAT-I gene KO mice (GM3 lacking mice) is enhanced, demonstrating that GM3 is a negative regulator of insulin signaling. On the other hand, we have reported that GM3 promotes malignant phenotype of 3LL Lewis lung carcinoma cells. Thus, GM3 is a key molecule in diabetes and cancer, so that we are interested in the regulation of SAT-I activity. In this study, we have investigated the role(s) of N-linked glycans of SAT-I in CHO cells. Using SAT-I antibody produced with the C-terminal 51 amino acids as the antigen, we have found that mouse SAT-I (mSAT-I) has N-linked glycans of complex or hybrid type, both of which are resistant to EndoH. The mSAT-I has three potential N-glycosylation sites (N-X-T/S; 180N, 224N, 334N). By examining the mutants replaced asparagine with glutamine (N180Q, N224Q, N334Q), we have identified that all of three sites are N-glycosylated. The amounts of N-glycans of complex or hybrid type in the SAT-I-N180Q mutant were decreased remarkably compared with those in the wild-type, nevertheless total amount of SAT-I protein unchanged. On the other hand, N334Q mutation in mSAT-I caused a reduction of total SAT-I protein by 50% of the wild type. We also examined sub-cellular localization of these three mutants with immunofluorescence microscopy. In the cells expressed N334Q mutants, ERs were transformed, and SAT-I-N334Q mutants were co-localized with ER marker proteins, suggesting that N334Q mutation causes an acceleration of degradation of SAT-I. Next, we examined the GM3 synthesis activity of SAT-I mutants (N180Q, N224Q, N334Q, and N180, 224, 334Q), and found that the single N to Q mutants exhibited the 10~30% activities compared with that of wild-type, whereas the triple mutant abolished the activity almost completely. Thus, we could suggest specific and distinct

functions of each N-linked glycans in mSAT-I, now focusing on the role of N-glycans, which might directly involve its activity and stability.

(112) **Cloning and Characterization of A Novel Human *N*-Acetylglucosaminyltransferase Homologous to GnT-V (GnT-IX)**

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The biological roles of *N*-linked oligosaccharides on glycoproteins are thought to play a role in the interaction of terminal glycan structures and their receptors. The diversity and avidity of the terminal structures are, however, regulated by the core structure of *N*-glycans. In vertebrates, six different *N*-acetylglucosaminyltransferases (GnT-I through GnT-VI) are involved in the biosynthesis of the highly branched complex-type *N*-glycan core stucture. A β1,6GnT, GnT-V catalyzes the transfer of GlcNAc from UDP-GlcNAc to the core α1,6-mannose arm via a β1,6-linkage, forming the tri- and tetraantennary complex-type *N*-glycans. When the GnT-V gene was cloned, no homologous genes were evident, suggesting that GnT-V has a unique evolutionary origin. The GnT-V ortholog gene (*gly-2*) was recently identified in *Caenorhabditis elegans*, and there is no homologous gene in this organism. However, we now identified a novel β1,6GnT cDNA by a BLAST search using the amino acid sequence of human GnT-V as a query. The full-length sequence was determined by a combination of 5'-rapid amplification of cDNA end analysis and a further database search. The open reading frame encodes a 792 amino acid protein with a type II membrane protein structure typical of glycosyltransferases. It appears to consist of an N-terminal cytoplasmic tail, a transmembrane domain, a stem region, and a C-terminal catalytic domain, and the entire sequence identity to human GnT-V is 42%. When pyridylaminated agalacto biantennary *N*-linked oligosaccharide was used as an acceptor substrate, the recombinant enzyme of the gene (designated as GnT-IX) exhibited β1,6GnT activity not only to the α1,6-linked mannose arm but also to the α1,3-linked mannose arm of *N*-glycan, forming a unique structure that has not been reported to date. Northern blot analysis showed that the GnT-IX gene is exclusively expressed in the brain, while the GnT-V gene is expressed ubiquitously. These results suggest that GnT-IX is responsible for the synthesis of a unique oligosaccharide structure in the brain.

(113) **Characterization of the Apoptotic Effects of N-Acetylmannosamine (ManNAc) Analogs in Jurkat (Human T-Lymphoma-Derived) Cells**

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Metabolic substrate-based sialic acid engineering is based on ability of exogenously-supplied analogs of N-acetylmannosamine (ManNAc) to enter a living cell, intercept the sialic acid biosynthetic pathway, and be metabolically incorporated into cellular glycoconjugates in the form of the corresponding non-natural sialic acid. This technology allows the cell surface to be endowed with novel physical and chemical properties that hold significant potential value for basic research, biomedical, and biotechnological applications. A number of challenges must be overcome before sialic acid engineering achieves large scale adoption; one issue is the decreased cell viability and cell death that results from the treatment of cells with ManNAc analogs bearing certain chemical and physical modifications. The purpose of this study is to investigate the molecular basis of ManNAc analog-induced growth inhibition and toxicity in order to devise strategies to avoid these deleterious effects. Ultimately, if these effects are understood in sufficient detail, it may be possible to direct them specifically to certain cell types, such as cancer cells, and thereby develop novel therapies for disease. In this study, we investigated a small panel of ManNAc analogs bearing various modifications on the hydroxyl groups as well as different substitutions at the N-acyl position. Jurkat (human T-lymphoma-derived) cells were incubated with the analogs and changes in cell viability were measured by using the trypan blue dye exclusion assay and Coulter counting. Decreased cell viability upon exposure of cells to analog was found to depend on the exact structural modification present and determined to be the result of the induction of apoptosis as shown by using DNA ladder formation and the Annexin V-FITC detection method. Further analysis showed that acetylated ManNAc analogs induce the activation of caspase 3 which is a key mediator of apoptotic cell death in mammalian cells. In addition, the induction of ManNAc analog-induced apoptosis corresponds to decreased metabolic flux through the sialic acid pathway and to changes in the expression of genes related to sialic acid biosynthesis. Interestingly, similar changes in gene expression, specifically the downregulation of sialyltransferases and the upregulation of sialidase, occurs with other apoptosis-inducing agents that do not directly impact sialic acid metabolism.

(114) **Potential Mechanism for Altered Sialylation in Cystic Fibrosis Airway Cells.**

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It has previously been shown that primary and immortalized cystic fibrosis (CF) airway epithelial cells have decreased sialic acid (NeuAc) content on membrane glycoproteins, and that it is dependent upon the expression of wild type cystic fibrosis transmembrane conductance regulator (wtCFTR), the protein mutated in CF (1). It is hypothesized that this altered glycosylation can contribute in part to the pathophysiology of lung disease in CF. To elucidate the mechanism of wtCFTR-dependent cell surface sialylation, the biochemical and molecular characteristics of endogenous enzymes involved in sialylation were examined in four well-characterized airway epithelial cell lines: IB3 (CFTR genotype: <FONTFACE="SYMBOL"SIZE="-1"DF508/W1282X), C38 (IB3 cells transfected with and expressing wtCFTR cDNA) and CFT43 (<FONTFACE="SYMBOL"SIZE="-1"DF508/<FONTFACE="SYMBOL"SIZE="-1"DF508) and BEAS2B (wild type, non-CF). First, these cell lines were confirmed to have wtCFTR-dependent sialylation: IB3 and CFT43 cells had 51% and 69% less sialic acid than C38 and BEAS2B cells, respectively ($p<0.05$). Next, using asialofetuin as substrate to transfer [^{14}C]NeuAc from CMP-[^{14}C]NeuAc, the activity of sialyltransferases of the CF IB3 cells were actually significantly higher than the activity of C38 cells (41.0 v. 22.7 pmol/mg protein/h, $p<0.002$) whereas there was no significant difference in transferase activity between CFT43 and BEAS2B extracts (53.0 v. 65.8 pmol/mg protein/h, $p>0.05$). To study possible transcriptional changes in glycosyltransferases based on wtCFTR expression, cDNA microarrays (2) of the genes involved in cell surface sialylation were performed. The mRNA for ST3Gal II was increased approximately 2-fold in IB3 cells compared to C38 cells ($p<0.05$) whereas CFT43 cells had 80% less and 20% more ST6GalNAc IV and ST6GalNAc V transcripts, respectively, than BEAS2B ($p<0.05$). There were no significant differences in other sialyltransferases detected on microarray. Therefore, decreased activity or decreased expression of sialyltransferases alone cannot adequately explain hyposialylation of all CF cells. To address the possibility that mislocalization of sialyltransferases could be responsible for decreased cell surface NeuAc, as previously proposed (3), experiments utilizing double immunofluorescent confocal microscopy were performed. In a series of experiments localizing two *N*-linked sialyltransferases in relation to the ER/Golgi markers GS28 and GS27 (BDBiosciences), it was found that ST6Gal I (4) was localized to an area corresponding to the Golgi compartment in IB3 and C38 cells and a perinuclear compartment overlapping with the Golgi in CFT43 and BEAS2B cells; ST3Gal III was found to be located in discrete vesicles in the trans-Golgi network and post-Golgi compartments in all cells. Both sialyltransferases were located in a more proximal position to the nucleus in the CF cells compared to the non-CF cells. These results support the hypothesis that a mislocalization of glycosyltransferases may be responsible for the altered glycosylation of cell surface glycoconjugates on CF airway epithelial cells (5). Experiments using electron microscopy and subcellular centrifugation are being performed to confirm these findings.

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(115) **Molecular Cloning and Identification Of 3'-Phosphoadenosine 5'-Phosphosulfate Transporter**

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Glycosylation, phosphorylation and sulfation are essential post-translational alterations of glycoproteins, proteoglycans, and glycolipids for normal growth and development. For sulfation, an activated form of sulfate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), is used as a common sulfate donor. Since sulfation occurs in the lumens of the endoplasmic reticulum and Golgi apparatus, PAPS must be translocated from the cytosol into the Golgi lumen through a specific transporter localized in the microsomal membrane. Although a specific PAPS transporter is present in Golgi membrane, no study has reported the corresponding gene.

We have identified a novel human gene encoding a PAPS transporter, which we have named *PAPST1*, and the *Drosophila melanogaster* ortholog, *slalom (sll)* . The amino acid sequence of PAPST1 (432 amino acids) exhibited 48.1 % identity with SLL (465 amino acids), and hydropathy analysis predicted the two to be type III transmembrane proteins. The transient expression of PAPST1 in SW480 cells showed a subcellular localization in Golgi membrane. The expression of PAPST1 and SLL in yeast *Saccharomyces cerevisiae* significantly increased the transport of PAPS into the Golgi membrane fraction. The apparent K_m values of PAPST1 and SLL were estimated to be 0.8 μM and 1.2 μM, respectively. No difference was observed among PAPST1, SLL, and mock in the transport of other nucleotide-sugars. The gene expression of *PAPST1* in human tissues was analyzed using real-time PCR. In human tissues, *PAPST1* is highly expressed in the placenta and pancreas, and present at lower levels in the colon and heart.

Drosophila melanogaster is a well-established model for genetic analysis. To elucidate the importance of the PAPS transporter to the viability of *Drosophila Melanogaster*, an inducible *sll* RNA interference (RNAi) fly using the GAL4-UAS system was made. We made four *UAS-sll inverted repeat (IR)* fly lines, and then used *Act5C-GAL4* as a GAL4 driver to induce *sll* gene knock-down in all cells of the fly. The relative amount of *sll* transcript in the F_1 of the *UAS-sll IR* fly crossed with the *Act5C-GAL4* fly is reduced to approximately one-fifth of that in the F_1 of w^{1118} crossed with *Act5C-GAL4*, which corresponds to the wild type. All four lines of the F_1 of the *UAS-sll IR* fly crossed with *Act5C-GAL4* exhibited pupal lethality, and no fly developed into an adult. These results demonstrated that the PAPS transporter is essential for viability. It is known that the developmental signaling functions of cell surface heparan-sulfate proteoglycans are dependent on their sulfation states. The role of the PAPS transporter in proteoglycan synthesis and the signaling pathway should be evaluated in further investigations.

Mutations of some genes related to PAPS synthesis have been reported to be responsible for human inherited disorders. On the other hand, no genetic disorder has been associated with the subsequent PAPS transport pathway. Our findings provide insights into the significance of PAPS transport and post-translational sulfation.

(116) **New Glycolipid, Phosphatidylglucoside in Lipid Rafts Involved in Granulocytic Differentiation of HL60 Cells**

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Most glycosphingolipids (GSLs) are localized in the outer leaflet of the plasma membrane, forming microdomains. The microdomain concept evolved from numerous studies over the course of two decades. Four lines of study were particularly significant: Clustering of GSL, (ii) Detergent-insoluble properties of clustered GSLs, (iii) Association of signal transducers with complex of glycosylphosphatidylinositol (GPI)-anchored proteins, GSLs, and caveolin, and (iv) Polarized presence of microdomains at the apical cell surface. Mixtures of microdomains are variously termed DIM, glycolipid enriched membrane (GEM), caveolae, or raft. GSLs in microdomains have multiple functions (e.g., toxin receptors, cell adhesion, cell growth modulators, and signal transduction initiators. Interestingly, Src-family kinases in the inner leaflet of the microdomain can be activated via surface GSL domains. Since there is enormous molecular diversity among membrane lipids, the existence of non-GSL lipid domains is quite possible. Despite the existence of many studies dealing with the characterization of membrane lipid domains, the precise composition of these domains is still not fully elucidated. When we analyzed the lipid composition of sphingolipid-deficient T-cells from sptlc2 knockout mice, we observed the presence of an unidentified lipid. We proceeded to isolate and characterize this novel glycolipid from HL60 cells and categorized this phosphatidylglucoside (PhGlc) as a new member of the lipid microdomain. To assess the roles of PhGlc, we generated a recombinant, monovalent Fab antibody, rGL-7, reacting preferentially with PhGlc. The treatment with rGL-7 induced differentiation of HL60 cells revealed by appearance of NBT-positive cells together with CD38 expression and c-Myc down-regulation. To determine the molecular mechanisms underlying early stages of signal transduction, we stimulated HL60 cells with rGL-7 and examined the protein tyrosine phosphorylation pattern. rGL-7 treatment induced rapid tyrosine phosphorylation of Src family protein kinases Lyn and Hck. Reduction of endogenous cholesterol after application of methyl-β-cyclodextrin suppressed rGL-7-stimulated tyrosine phosphorylation. We found co-localization of the phosphorylated proteins and PhGlc in the Triton X-100 insoluble, light buoyant density fraction following sucrose gradient ultracentrifugation of HL60 cell lysates, suggesting an involvement of PhGlc-based microdomain in the GL-7 signaling. On the other hand, sitimulation of known components of microdomains such as sphingomyelin and ganglioside GM1 with the corresponding antibodies did not induce the differentiation as well as tyrosine phosphorylation in HL60 cells. These results show that PhGlc constitutes a new lipid-signaling domain and the glucose residue of PhGlc is critical for organization of carbohydrate-dependent signaling domain involved in cellular differentiation of HL60 cells.

(117) **Studies on the Interaction between Autocrine Motility Factor Receptor and Mouse Peptide: N-glycanase**

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Cytoplasmic peptide: *N*-glycanase (PNGase) is an enzyme involved in the deglycosylation of glycoproteins and glycopeptides. A yeast two-hybrid screening showed that mouse PNGase interacts with several proteins, including autocrine motility factor receptor (AMFR), mHR23B, mY33k, mS4 and ubiquitin (1). However, the yeast homolog of PNGase only interacts with Rad23p (homolog of mHR23B) (2). This suggests that PNGase may function in several processes in mammalian cells. AMFR, a multimembrane spanning protein, has been shown previously to be a cell surface receptor mediating tumor invasion and metastasis. Interestingly, it was also identified as an ER membrane-anchored RING finger-dependent E3 ubiquitin ligase. It is the first mammalian ER resident E3 ligase to be reported (3). AMFR ubiquitinates the T cell antigen receptor CD3 δ -subunit (3) and apoB (the essential protein component of atherogenic very low density and low density lipoproteins) (4) for proteosomal degradation. Physical interaction of mPNGase with AMFR has been confirmed using GST pull-down assays. Immunofluorescence in COS1 cells showed the colocalization of the two proteins. Co-immunoprecipitation studies on mPNGase and AMFR are currently being carried out. There are two possibilities to explain the interaction of these two proteins. One is that it is possible that AMFR is responsible for the degradation of mPNGase. The other possibility is the two enzymes work together in the degradation progress of misfolded glycoprotein. Efforts to test these two hypotheses are in progress. (Supported by NIH Grant GM33184 to WJL).

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(118) **Five Genes Involved in Biosynthesis of the Pyruvylated Galβ1,3-Epitope In *Schizosaccharomyces pombe* N-Linked Glycans**

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The N-linked galactomannans of *Schizosaccharomyces pombe* have pyruvylated Galβ1,3- (PvGal) caps on a portion of the Galα1,2- residues in their outer chains (Gemmill and Trimble (1998) *Glycobiology*, 8, 1087–1095). To investigate the biosynthesis of the PvGal moiety, *S. pombe* cells were mutagenized using ethylmethanesulfonate, and mutants defective in the synthesis of the negatively charged N-glycans were isolated by Q-Sepharose exclusion and failure to bind human serum amyloid P component, which acts as a lectin for terminal PvGal residues. Glycosylation mutants were characterized through lectin binding, exoglycosidase sensitivity, NMR spectroscopy, and saccharide compositional analysis. Restoring the cell surface negative charge by complementation with an *S. pombe* genomic library resulted in identification of five genes, designated *pvg1–pvg5*, involved in PvGal epitope biosynthesis. Pvg1p may be a pyruvyltransferase, since NMR of *pvg1*$^-$-mutant N-glycans revealed the only difference from those of wild type to be the absence of pyruvyl moieties. Pvg2p–Pvg5p are crucial for attachment of the Galβ1,3- residue that becomes pyruvylated. Pvg3p is predicted to be a member of the β-galactosyltransferase family which includes over 90 members conserved from plants to man. Chimeric Pvg3p-green fluorescent protein (GFP) yielded punctate staining consistent with Golgi localization. The putative functions of Pvg1p and Pvg3p suggest that the Galβ1,3- is added to the maturing galactomannan chains and then subsequently pyruvylated *in situ*, rather than *en bloc* addition of PvGalβ1,3- to outer chain Galα1,2- residues. Pvg4p-GFP targeted to the nucleus and its sequence contains a MADS box DNA-binding and dimerization domain. However, expression analysis by endpoint RT–PCR indicated that all five genes (plus actin control) are expressed in all five mutants. Therefore, Pvg4p does not appear to solely control expression of any of the other four proteins, and other genes involved in the expression of the PvGal epitope may await discovery. It is possible that Pvg2p and/or Pvg5p may form part of an enzyme complex that assembles the PvGal epitope. Knockout studies and experiments to further elucidate the relationship between these proteins are ongoing. While a functional role for the PvGal epitope in *S. pombe* remains unclear, it was found to be nonessential for either cell growth or mating under laboratory conditions.

(119) **Interaction between An OT Subunit , Wbp1p, and Yeast Protein Kinase C: Insight into the Functional Relationship**

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Oligosaccharyl transferase (OT) is a multisubunit enzyme complex that catalyzes the en bloc transfer of oligosaccharides from Dol-PP onto the nascent polypeptide chain. In the yeast Saccharomyces cerevisiae, 9 different subunits of oligosaccharyl transferase (OT) have been cloned and identified. However, at present little is known about the function of each of the subunit. Recently several of the OT subunits have been found to participate in biological processes other than the N-glycosylation per se and therefore were proposed to have multiple functions. Specifically, Stt3p was found to interact with Kre5p and Kre9p in a synthetic lethal screen and was proposed to be involved in Pkc1p regulated cell wall β -1,6 glucan synthesis (Chavan, M.and Lennarz, W.J., manuscript in preparation). Moreover, Wbp1p was found to interact with one of the Sec61p proteinaceous channel component, Sss1p, which implies that it may play a role in coordination of co-translational protein translocation and N-glycosylation (1).

Several of OT subunits were found to interact with protein kinase C (Pkc1p) in a yeast two-hybrid screen study using the lumenal domains of OT subunits as bait proteins (2). However, this result is difficult to interpret because Pkc1p is believed to be a cytoplasmic protein rather than a lumenal protein. Due to the difficulty in utilizing the yeast two-hybrid screen in the case of membrane proteins, we utilized the split-ubiquitin system, a newly developed approach to detect protein-protein interactions in vivo (3), to study the possible interaction of full length Wbp1p with Pkc1p. A strain which contains the sequence of Cub-PLV (C-terminal of ubiquitin linked with protein A-LexA-Vp16 as reporter gene) at the C-terminus of WBP1 in its chromosome copy was transformed with a series of plasmids having the Nub (N-terminal of ubiquitin) attached to the C-terminus of PKC1. The β -gal analysis as well as growth assays revealed that Wbp1-Cub-PLVp interacts with Pkc1-Nubp. Furthermore, cleavage of the reporter gene, which is the indicator of the interaction between the two proteins tested, was also detected by Western blot analysis using anti-LexA antibody. This interesting result establishes an interaction between the Wbp1p subunit of OT and Pkc1p suggesting an interrelationship between N-glycosylation and the MAP kinase pathway in yeast. Furthermore, another genetic result : overexpression of *WBP1* gene was found to rescue the growth defect caused by overexpression of a Pkc1 active site mutant also supports these findings (2). Co-immunoprecipitation experiments as well as additional biochemical studies are underway to resolve this interesting interrelationship between an OT subunit and Pkc1p. (Supported by NIH Grant GM 33185 to WJL) References 1. Scheper W, Thaminy S, Kais S, Stagljar I, Romisch K, J. Biol. Chem., 2003, in press. 2. Park, H., and Lennarz, W. J., (2000) Glycobiology 10, 737-747. 3. Stagljar, I., Korostensky, C., Johnsson, N., and te Heesen, S. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 5187-5192.

(120) **Withdrawn**

(121) **A Novel Interaction between GPT (GlcNAc-1-P Transferase) and Lec35p**

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GPT (GlcNAc-1-P transferase) initiates synthesis of lipid-linked oligosaccharide (LLO) by transferring GlcNAc-1-P from UDP-GlcNAc to dolichol-P, forming GlcNAc-PP-Dol. Normally, this leads to synthesis of $Glc_3Man_9GlcNAc_2$-PP-Dol. Previous studies have shown that strong overexpression of GPT (eg. 10-fold) in CHO-K1 cells results in the accumulation of the LLO $Man_5GlcNAc_2$-PP-Dol in place of $Glc_3Man_9GlcNAc_2$-PP-Dol. It has been hypothesized that excessive consumption of Dol-P by overexpressed GPT results in less formation of Man-P-Dol (MPD) and Glc-P-Dol (GPD), which also require Dol-P for synthesis. Reducing the MPD and GPD would then be expected to inhibit the extension of $Man_5GlcNAc_2$-PP-Dol. This hypothesis predicts that GPT overexpression should (a) increase the molar quantity of LLO and (b) reduce the molar quantities of MPD and GPD. However, it had also been reported previously that the quantities of [^{3}H]mevalonate-labeled MPD and GPD are not highly reduced in GPT overexpressing cells, and that the amounts of [^{3}H]mannose-labeled LLO are not significantly increased.

We have re-examined this hypothesis by analysis of LLO, MPD, and GPD with fluorophore-assisted carbohydrate electrophoresis (FACE), to accurately determine the molar quantities of these molecules. FACE revealed that the molar quantity of LLOs increased only 2-fold by GPT overexpression. Comparison with another $Man_5GlcNAc_2$-PP-Dol producing line, Lec15, showed that the 2-fold increase was related to the oligosaccharide defect, not GPT overexpression. Further, FACE showed that MPD and GPD were produced at essentially normal levels in GPT overexpressers. We then tested the effects of tunicamycin (TN), an inhibitor of GPT. Mild TN treatments decreased the molar quantity of LLO, but did not restore extension of $Man_5GlcNAc_2$-PP-Dol to $Glc_3Man_9GlcNAc_2$-PP-Dol. Thus, $Man_5GlcNAc_2$-PP-Dol accumulation is not readily explained by excessive consumption of Dol-P.

An alternative hypothesis is that GPT itself inhibits addition of the 6th mannosyl residue and the 1st glucosyl residue. Specifically, GPT might interfere with the function of Lec35p, which is necessary for utilization of both MPD and GPD in LLO synthesis. If so, overexpression of Lec35p should reverse the effects of overexpression of GPT. We tested CHO-K1 transfectants with Lec35p under a regulated promoter, and also expressing different amounts of GPT. FACE showed that the ratio of $Man_5GlcNAc_2$-PP-Dol to $Glc_3Man_9GlcNAc_2$-PP-Dol was dependent upon the ratio of GPT to Lec35p, not the absolute amounts of GPT or Lec35p. Additional experiments confirmed that the phenotype of cells overexpressing GPT was very similar to the phenotype of cells lacking Lec35p. Therefore, GPT overexpression appears to cause accumulation of $Man_5GlcNAc_2$-PP-Dol by interfering with Lec35p, not by excessive consumption of Dol-P. This suggests a regulatory interaction between the two proteins.

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(122) **ER Stress-Dependent Transcription of Human Genes Represented on the *Consortium for Functional Glycomics* Glyco-gene Chip**

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The "Unfolded Protein Response" (UPR) is an umbrella term that refers to a set of signaling events resulting from stress in the lumenal compartment of the endoplasmic reticulum (ER). ER stress is typically caused by excessive protein misfolding. Originally considered mainly a response to external agents that perturb ER function, the mammalian UPR is now clearly understood to be involved in both physiological and pathological events. The ER plays a critical role in N-glycan synthesis and other forms of glycosylation, and recent hypotheses have implicated the UPR in the cell's ability to respond to programmed increases in glycoprotein synthesis. Thus, in addition to increased transcription of genes encoding ER chaperones and enzymes that participate in the folding of nascent glycoproteins, the UPR might also be expected to activate genes involved in synthesis of glycan precursors, transfer of glycans to protein, and glycan remodeling.

As a test of this hypothesis, microarray analysis of approximately 1000 human genes on the *Consortium For Functional Glycomics* Glyco-gene chip, with functions relating to glycan synthesis and recognition, is being performed with mRNA isolated from human fibroblasts subjected to a series of highly characterized ER stresses. Initial analyses indicate that genes involved in the synthesis of glycans by the ER are among those that are significantly stimulated. Therefore, these results support the hypothesis that protein glycosylation in a *bona fide* target of the UPR.

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(123) **The Identification and Characterization of the *Saccahromyces cerevisiae* ORF YJR013Wp, A Highly Conserved Essential Protein of GPI-Anchor Synthesis, Homologous to the Mannosyltransfersase Pig-Mp**

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GPI-anchors are essential structures of all known eukaryotic classes, which attach proteins to the outer leaf of the apical cell surface. This is done using a short carbohydrate chain to link the membrane-bound lipid inositol moiety to the protein. In many cells the GPI-anchor serves to target the protein to lipid rafts called caveolae. Many GPI-anchors, however, do not receive proteins and yet reside on the cell surface. Elucidating additional roles GPI-anchors play in cellular biochemistry has spurred interest in defining the GPI-anchor synthetic pathway. Using the sophisticated bioinformatic program PROBE our group identified the ORF YJR013W as a potential glycosyltransferase either of N-linked glycan or GPI-anchor synthesis. Recently, a group in Japan (Maeda *et al.* (2001) *EMBO J.*, 20, 250-261) have identified YJR013W as having 35% amino acid homology to human Pig-Mp, the first mannosyltransferase in GPI-anchor synthesis. Their report as well as others (Tekaia *et al.* (2000) *FEBS Lett.*, 487, 31-36) confirms our data that YJR013Wp is 98 amino acids longer than indicated by the annotated *Saccharomyces cerevisiae* database. Current experiments confirm the essential nature of YJR013Wp, determine the exact length of its mRNA transcript, attempt to determine the orientation of the DXD motif with respect to the ER lumen/cytosol, and are aimed at verifying whether GDP-Man or Dol-P-Man acts as the sugar donor for YJR013Wp as well as Pig-Mp.

(124) **New Pkc1p-Like Proteins in Yeast: Possible Role in Glycosylation and Folding of N-Linked Glycoproteins**

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N-linked protein glycosylation is the most common post-translational modification of secretory proteins in eukaryotes. The key step in the glycosylation process, occuring at the luminal face of the ER membrane, targets a pre-assembled oligosaccharide chain to transfer from the lipid carrier to asparagine residues of nascent polypeptide chains via the oligosaccharyltransferase complex (OT). OT consists of at least eight different subunits. Both genetic and biochemical studies in yeast demonstrated that Stt3p is the most conserved subunit of the OT and it is involved in peptide recognition and/or catalysis (1). In the two-hybrid library screen Pkc1p, previously known to be involved in the MAP kinase signal transduction pathway that modulates yeast cell wall synthesis, interacts with the luminal part of several OT subunits, including Stt3p. In addition, Pkc1p modulates OT activity in microsomes from wild-type and pkc1 null by an unknown mechanism, as assayed by glycosylation of a simple acceptor peptide (2). In eukaryotic cells, the folding of N-linked glycoproteins that are destined to be secreted or membrane bound takes place in ER. The accumulation of misfolded glycoproteins triggers an ER-stress response through an ER-resident transmembrane kinase, Ire1. This response has at least two distinct components. The first component, known as the unfolded-protein response (UPR) consists of the transcriptional induction of genes which promote the folding of newly synthesized proteins in the ER. The second component consists of a profound and rapid repression of protein synthesis. It is associated with polysome disassembly and correlates with increased phosphorylation of eIF2a. In yeast data bank screening we found 6 homologs of Pkc1p. Among these, one of these shares homology to a Caenorhabditis elegans gene. This gene encodes a predicted type I transmembrane protein with a cytoplasmic domain similarity to eIF2a kinases and a lumenal domain that shares similarity to mammalian Ire1p (3). The localization and function of these homologs is unknown. In our work we are focusing on tagging these homologs in order to identify their cellular localization and function in N-glycosylation and folding mechanisms. This work is supported by NIH Grant, GM 33185. References: 1) Yan Q. and Lennarz W.J., JBC 49, 47692-47700, 2002. 2) Park H. and Lennarz W.J. , Glycobiology 7, 737-744, 2000. 3) Harding H.P. et al., Nature 397, 271-274, 1999.

(125) **Molecular Cloning and Characterization of a Novel Human β1,4-N-Acetylgalactosaminyltransferase, β4GalNAc-T3, Responsible for the Synthesis of N,N'-Diacetyllactosediamine, GalNAcβ1-4GlcNAc**

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We found a novel human glycosyltransferase gene carrying a hypothetical β1,4-glycosyltransferase motif during a Blast search, and cloned its full-length open reading frame using the 5' rapid amplification of cDNA ends method. It encodes a type II transmembrane protein of 999 amino acids with homology to chondroitin sulfate synthase in its C-terminal region (GenBankTM accession number AB089940). Its putative orthologous gene was also found in mouse (accession number AB114826). The truncated form of the human enzyme was expressed in human embryonic kidney HEK293T cells as a soluble protein. The recombinant enzyme transferred N-acetylgalactosamine (GalNAc) to N-acetylglucosamine (GlcNAc) β-benzyl. The product was deduced to be GalNAcβ1-4GlcNAcb-benzyl based on mass spectrometry and NMR spectroscopy. We renamed the enzyme β1,4-N-acetylgalactosaminyltransferase-III (β4GalNAc-T3). β4GalNAc-T3 effectively synthesized N,N'-diacetylgalactosediamine, GalNAcβ1-4GlcNAc, at non-reducing termini of various acceptors derived not only from N-glycans, but also from O-glycans. Quantitative real-time PCR analysis showed that its transcript was highly expressed in stomach, colon and testis. As some glycohormones contain N,N'-diacetylgalactosediamine structures in their N-glycans, we examined the ability of β4GalNAc-T3 to synthesize N,N'-diacetylgalactosediamine structures in N-glycans on a model protein. When fetal calf fetuin treated with neuraminidase and β1,4-galactosidase was utilized as an acceptor protein, β4GalNAc-T3 transferred GalNAc to it. Furthermore, the majority of the signal from GalNAc disappeared on treatment with glycopeptidase F. These results suggest that β4GalNAc-T3 could transfer GalNAc residues, producing N,N'-diacetylgalactosediamine structures at least in N-glycans, and probably in both N- and O-glycans.

(126) **The O-Glcnac Modification of Proteins Interferes with Signaling by Pka and Cdk5 Dependent Phosphorylation in Neurons**

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N-acetylglucosamine linked O-glycosidically to serine and threonine residues of proteins (O-GlcNAc) is a posttranslational modification of proteins of the cytosol, the nucleus and of the cytosolic tail of transmembrane proteins. We have previously shown that the O-GlcNAc level of proteins from neurons in culture respond reciprocally to inhibition or activation of some kinases by

treatment of cells with specific pharmacological agents. Vice versa, inhibition of O-GlcNAc hydrolase with the specific inhibitor PUGNAc results in increased O-GlcNAc levels of proteins. Extending these earlier studies we show that treatment of N2a neuroblastoma cells in culture with 1. PUGNAc and 2. the PKA activator dbcAMP paradoxically leads to an increase of the O-GlcNAc level of several proteins which is significantly higher than the treatment with PUGNAC alone. Addition of PUGNAc and dbcAMP in the reverse order did not result in enhanced O-GlcNAc levels. Since CDK5 (cyclin-dependent kinase 5) - an important neuronal kinase possibly implicated in the pathology of the Alzheimer´s disease - is a downstream target of PKA, we studied O-GlcNAc expression in the presence of the CDK5 specific inhibitor roscovitine. The same strong increase of the O-GlcNAc level of proteins was detected in the presence of PUGNAc and roscovitine as in the presence of PUGNAc and dbcAMP. Western blot analysis of immunoprecipitates showed that CDK5 itself is also O-GlcNAc modified suggesting that in addition to phosphorylation the O-GlcNAc modification may regulate the activity of CDK5. These observation provide further evidence that signaling mechanisms are modulated by a cross talk between phosphorylation and O-GlcNAc glycosylation of proteins.

(127) **Endocytosis of Antithrombin III by Endothelial Cells.**

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Antithrombin III is the major plasma inhibitor of coagulation enzymes and is the archetypal heparin-binding protein. An uncommon pentasaccharide sequence within heparin and heparan sulfates is responsible for specific and high affinity binding involved in the activation of antithrombin. Thus, the interaction of antithrombin with vascular endothelial cells, which are rich in heparan-sulfate proteoglycans, would appear to be an important means for regulating the concentration and activity of this plasma glycoprotein. We demonstrate that fluorescently labeled antithrombin can be used as a marker for endothelial cells. For the first time, we demonstrate that antithrombin is endocytosed by endothelial cells, and this endocytosis is not reliant upon formation of complexes with proteases such as thrombin. Moreover, at physiological concentrations of antithrombin, internalization cannot be explained by a passive mechanism. The internalization and binding of antithrombin to the surface of endothelial cells is not solely mediated by the high-affinity binding to the pentasaccharide sequence.

(128) **The Presence of the HNK-1 Epitope without Sulfate in Mouse Kidney**

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The HNK-1epitope is a unique modification of glycans, in which 3-sulfo-glucuronyl residue is attached to lactosamine structures 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. Several years ago, we cloned the enzymes responsible for the biosynthesis of this epitope, two glucuronyltransferases (GlcAT-P and GlcAT-S) and one sulfotransferase (Sulfo-T). During the course of a study using these molecular tools, we found an interesting observation. Thus, GlcAT-P mRNA was expressed almost exclusively in the brain. In contrast, GlcAT-S mRNA was highly expressed in the kidney as well as in the brain. Sulfo-T mRNA, which is expressed almost ubiquitously among various tissues, was not expressed significantly in the kidney. These results suggested that the HNK-1 epitope without sulfate may be present in the kidney. This was tested using two different monoclonal antibodies, one (HNK-1) requires sulfate on the glucuronyl residue for the binding and the other (M6749) does not. Histochemical studies of mouse kidney with these mAbs indicated that M6749 mAb stained specifically the proximal tubules of the cortex, while HNK-1 mAb did not stained the kidney at all, confirming our hypothesis. Upon western blot analysis of the membrane fraction of the kidney with M6749 mAb, two positive bands were detected; one major band at the position of 80kDa protein and a minor one of 140kDa, both of which disappeared completely after digestion with N-glycanase F, indicating that these proteins bear the HNK-1 epitope without sulfate on N-glycan chains. The following proteomics-type analysis using MS suggested that the 80kDa band contained meprin, a zinc metalloprotease with 10 potential N-glycosylation sites. This is the first report indicating the presence of the HNK-1 epitope in the kidney.

(129) **The Recombinant Rat 175 kDa Hyaluronan Receptor for Endocytosis (HARE) Mediates the Uptake of Hyaluronan, Dermatan Sulfate and the Chondroitin Sulfates.**

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The clearance of hyaluronan (HA) and chondroitin sulfate (CS) from lymph fluid and blood in mammals is mediated by the HA Receptor for Endocytosis (HARE), which is expressed in the sinusoidal endothelial cells of the liver, spleen and lymph nodes. Two isoforms of rat and human HARE (175/300 kDa and 190/315 kDa, respectively) are present in these tissues. The small rat and human HARE proteins are not encoded directly by mRNA, but are derived by proteolysis from larger precursors. We have characterized the specificity and function of the 175 kDa HARE, expressed in the absence of the larger 300 kDa species, in stably transfected SK-Hep-1 cells. The HARE cDNA was fused with the leader sequence of the Ig kappa-chain to allow correct orientation of the protein in membranes. The recombinant rHARE contained ~25 kDa of N-linked oligosaccharides and, like the native protein, was able to bind HA in a ligand blot assay, even after de-N-glycosylation. SK-HARE cell lines demonstrated specific ^{125}I-HA endocytosis, receptor recycling, and delivery of HA to lysosomes for degradation. Dissociation constants for the binding of the 175 kDa HARE to HA (Mw ~133 kDa) were about 4 nM with 160,000-220,000 HA-binding sites per cell. Based on competition studies, the 175 kDa rHARE binds and mediates endocytosis of HA, dermatan sulfate and chondroitin sulfates A, C, D, and E, but does not recognize chondroitin, heparin, heparan sulfate, or keratan sulfate. Surprisingly, recognition of glycosaminoglycans other than HA by native or recombinant HARE was temperature-dependent. Although competition was observed at 37°C, none of the other glycosaminoglycans competed for ^{125}I-HA binding to SK-HARE cells at 4°C. Monoclonal antibody-174, which was raised against the native rat 175 kDa HARE, showed a similar temperature-dependence in its ability to block HA endocytosis at 37°C, but not binding at 4°C. The results confirm that the 175 kDa rHARE does not require the larger HARE isoform to mediate endocytosis of multiple glycosaminoglycans. The results also suggest that temperature-induced conformational changes may alter the glycosaminoglycans specificity of HARE. (supported by NIH grant GM35978)

(130) **α2-6 Sialylation Regulates β1 Integrin Function**

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An altered level of α2-6-linked sialic acid is one of the most common carbohydrate changes observed in neoplastic disease. For example, colon adenocarcinomas consistently express elevated levels of both α2-6 sialic acid and ST6Gal I, the cognate sialyltransferase mediating the α2-6 linkage. Furthermore, elevated sialic acid levels correlate with metastasis and a poorer prognosis in these tumor types, strongly suggesting a functional role for α2-6 sialylation in cancer progression. Nonetheless, the protein targets for this elevated sialylation have remained largely unexplored. Our previous work in colon epithelial cells has shown that ras dramatically regulates ST6Gal I expression, and in turn α2-6 sialylation, of β1, but not β3 or β5, integrin cell adhesion receptors (Seales et al. Oncogene in press). Therefore, one mechanism of oncogenic ras-mediated cell transformation might involve dysregulation of β1 integrin function via altered sialylation. In support of this notion, ras-mediated changes in β1 sialylation were correlated with altered cell adhesion to collagen (a β1 ligand). In contrast, no changes were observed in cell adhesion to vitronectin (a β3 or β5 ligand), suggesting that β1, but not other β integrins, is a functional target of ras-mediated sialylation. However, ras is known to exert multiple effects on cell behavior. Therefore, we wanted to bypass the ras-signaling cascade and manipulate α2-6 sialylation directly via the ST6Gal I enzyme. In the present study, we stably overexpressed ST6Gal I in SW48 cells, a colon epithelial cell line which completely lacks endogenous sialyltransferase activity. ST6Gal I overexpressers show high α2-6 sialylation of β1 compared to undetectably sialylated β1 from control SW48 cells, confirming this integrin's role as a substrate for ST6Gal I. Neither β3 nor β5 integrins were detected in this cell line. Stable ST6Gal I overexpressers demonstrate elevated β1-mediated adhesion to collagen and laminin, effects which are completely reversed by cell surface treatment with the sialic-acid-cleaving enzyme, sialidase. These data are in agreement with our previous results showing that sialidase treatment of purified α1β1 integrins (which are α2-6 sialylated) led to reduced binding of these integrins to collagen. Since all of this work was performed in cultured cell lines, we next wanted to show a

true biological relevance for β1 sialylation in vivo. Given that oncogenic ras can regulate the level of β1 α2-6 sialylation in our cell lines, we anticipated that colon adenocarcinomas (which harbor both high rates of ras mutations and elevated ST6Gal I activity) should target the β1 integrin for elevated sialylation. We have indeed confirmed that β1, but not β3, integrins from carcinoma tissues carry elevated α2-6 sialylation relative to pair-matched control tissues. Immunofluorescent double-labeling has also confirmed the colocalization of β1 and α2-6 sialic acid in these tissues. In light of our in vitro and in vivo findings, we propose two main points. First, β1 integrins are targets for ras pathway-mediated α2-6 sialylation in vivo. Secondly, this α2-6 sialylation is a critical modulator of β1 integrin function.

(131) **Gel Electrophoretic and Mass Spectrometric Analysis of Beta-1 Integrin to Identify Occupied N-Linked Glycosylation Sites**

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Amongst the myriad of molecules found on the cell surface responsible for cell-cell and cell-extra cellular matrix interactions, is a family of large transmembrane glycoproteins called integrins. Divided into two sub-families, the alphas and betas, a functional integrin complex is composed of a non-covalent interaction between an alpha and beta subunit. The integrin with the most varied binding profile is beta-1 integrin, a 784 aa glycoprotein with 12 putative N-linked glycosylation sites. Since it has also been noted that oncogenic transformation of cells causes structural and functional changes in Beta-1 integrin N-linked glycans, complete characterization of the carbohydrate chains of this important cell surface glycoprotein, from wild type and transformed cells, would be insightful. In the present study the initial focus was to first determine which of the 12 putative N-linked glycosylation sites of Beta-1 integrin are actually occupied. HEK 293 cells were stably transfected with T-7 epitope tagged Beta-1 integrin using hygromycin B as a selection marker. The glycoprotein was then isolated over a T-7 tag antibody column. Purified Beta-1 integrin made in the HEK 293 cells was subjected to gel electrophoresis to separate the isoforms of the protein with different levels of glycosylation. The relevant bands were then thermolysin treated, extracted from the gel and analyzed by LC/MS using a QTOF. The determination of these sites paves the way for future experiments to completely characterize the oligosaccharides of Beta-1 integrin, as well as for site-directed mutagenesis to determine the activity and importance of each occupied N-linked site.

(132) **Cation Dependence of the 46kDa Mannose 6-Phosphate Receptor**

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There are two P-type lectins, the 46kDa mannose 6-phosphate receptor (MPR46) and the 300kDa insulin-like growth factor II/cation-independent MPR (MPR300), that exist in virtually all higher eukaryotes. The MPRs play an essential role in the biogenesis of lysosomes: the receptors recognize mannose 6-phosphate (M6P)-containing lysosomal enzymes in the trans Golgi network and transport them to acidified endosomes by interacting with the N-terminal VHS domain of the Golgi-localized, -ear-containing, ARF-binding proteins (GGAs), thereby diverting them from the secretory pathway. Previous crystallographic studies of the MPR46 in the presence of pentamannosyl phosphate (PMP) have identified eleven amino acids that are predicted to form hydrogen bonds with PMP: nine residues (Tyr45, Gln66, Asp103, Asn104, His105, Arg111, Glu133, Arg135, and Tyr143) interact with the terminal phosphorylated mannose and two amino acids (Asp43, Gln68) interact with the penultimate and prepenultimate mannose rings of PMP. Previous studies by Tong and Kornfeld demonstrated that the bovine MPR46 displayed a 4-fold enhanced binding affinity to the monosaccharide, M6P (Kd = 6uM) in the presence of $MnCl_2$ versus Kd = 25uM in the presence of EDTA. In contrast, studies by Junghans et al. and Baba et al. showed that MPR46 purified from human and porcine has no or little cation-dependence, respectively. To further investigate the cation dependence of the MPR46, a soluble, glycosylation-deficient bovine MPR46 was generated. The protein was purified from *Pichia pastoris* and subjected to quantitative binding studies with an endogenous substrate, β-glucuronidase. The results demonstrate that the bovine MPR46 displays a 250-fold higher binding affinity in the presence of the cation Mn^{2+} (10mM $MnCl_2$, Kd = 1.5 ± 0.2 nM) than in the presence of 10mM EDTA (Kd = 376.6 ± 102.8 nM). To directly determine the MPR46's binding affinity towards the cation Mn^{2+}, Electron Spin Resonance (ESR) has been employed and the preliminary data show that MPR46 has a Kd of 57uM towards the cation Mn^{2+} when probed in the presence of 100uM M6P. In addition, Asp103 is the only amino acid that coordinates the observed Mn^{2+} in the binding pocket and thus is proposed to account for the higher affinity of the bovine MPR46 towards its ligands in the presence of cation. To test this hypothesis, two Asp103 mutants, D103N and D103S, have been generated by site-directed mutagenesis and their binding affinity towards β-glucuronidase and the cation Mn^{2+} will be determined. (Supported by NIH grant DK42667 to N.M.D.)

(133) **Contact Inhibition of Cell Growth, and Loss of this Process, as Related to GM3- and FGF-Dependent Signaling through cSrc and Csk Activity in Microdomain: WI-38 vs. VA-13 Cells**

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Contact inhibition of cell growth, and its loss during tumorigenesis, are well-documented "common denominators" of many transformed cells. In view of recent developments regarding structure and function of microdomains, we investigated the topic with focus on microdomain structure and function. Ganglioside GM3, fibroblast growth factor receptor (FGFR), Src kinase, and tetraspanins CD9 and CD81 were highly enriched in low-density membrane fraction (GEM, prepared in Brij 98) of both human lung embryonic fibroblast cell line WI-38 (showing high degree of contact inhibition) and its SV40 virus transformant VA-13 (showing loss of contact inhibition). However, chemical quantities of CD9 and CD81 were much higher in WI-38. The following major functional differences were observed: (i) MAPK was strongly activated in response to FGF when GM3 was depleted by P4 treatment in sparse-growing WI-38, whereas there was no such FGF/GM3 effect on confluent WI-38. (ii) Both cSrc and MAPK activation were observed in VA-13 cells regardless of degree of FGF stimulation, or even in the absence of FGF stimulation. This response was consistent regardless of cell population density. (iii) Csk, the physiological inhibitor of cSrc, was much higher in WI-38 GEM. These findings indicate that microdomain organization in terms of association of GM3 with FGFR, and with cSrc/Csk, could be mediated by CD9 and CD81 in contact-inhibitable WI-38, whereas GM3 interaction with FGFR in VA-13 is not mediated by these tetraspanins. Thus, cSrc and MAPK activation in response to cell population density are lost in VA-13. This work was supported by NIH/ NCI Grant R01 CA80054 (to S.H.).

(134) **Subcellular Localization of Human Cosmc and T-Synthase**

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Human core 1β3-galactosyltransferase (Core 1 β3-Gal-T) (T-synthase) is a ~42 kDa subunit, dimeric enzyme responsible for synthesizing the core 1 O-glycan Galβ1-3GalNAc-α-1-Ser/Thr (T-antigen), which is also a precursor for many extended O-glycans in animal glycoproteins and mucins. We previously reported that expression of active T synthase requires the cooperation of a Core 1 β3-Gal-T Specific Molecular Chaperone (Cosmc) (Ju T. and Cummings R.D., 2002, *PNAS*, 99(26): 16613-16618). Cosmc is a regulatory factor in O-glycan biosynthesis pathway, since mutations in the *Cosmc* gene in human Jurkat and human tumor LSC cells prevent expression of active T-synthase, resulting in exposure of the Tn antigen (GalNAc-α-1-Ser/Thr). Many known chaperones important in the secretory pathway function in the endoplasmic reticulum (ER). Epitope-tagged Cosmc expressed in Hi-5 insect cells can bind ATP-Sepharose and is eluted with ATP, and has no T-synthase activity, consistent with a chaperone function of Cosmc. To explore Cosmc localization and possible chaperone functions in the ER, we examined the subcellular localization of both Cosmc and T-synthase. Recombinant YFP-tagged Cosmc expressed in CHO K1 cells displays peri-nuclear pattern under fluorescence microscope and co-localized with ER-tracker. Similarly, C-terminal HA–tagged Cosmc expressed in CHO K1 cells was stained with Alexa488 labeled anti-HA monoclonal antibody, co-localized with PDI, an ER marker. By contrast, C-terminal HPC-tagged T-synthase colocalized with a-mannosidase II, a medial-Golgi marker. HA-tagged Cosmc and HPC-tagged T-synthase co-expressed in CHO K1 cells showed that they localize in different compartments. These results indicate that Cosmc localizes in ER, whereas T-synthase localizes in medial-Golgi. A recombinant, soluble form of Cosmc containing an N-terminal epitope tag, is mostly secreted into the media, but it is still capable of slightly increasing T-synthase activity in 293T cells when it is cotransfected with a cDNA encoding the full-length T-synthase. By contrast, a soluble form of Cosmc with KDEL at the C-terminus is efficiently

retained in the ER and is more efficient in promoting activity of T-synthase. These results are consistent with the proposed function of Cosmc in the ER compartment to facilitate folding/activity of T-synthase. Interestingly, human Cosmc has 1 predicted N-glycosylation sequence near the C-terminus. Endoglycosidase digestion indicated that this site is partially glycosylated; however, mutation of this sequon eliminates N-glycosylation but does not affect the ability of the mutated Cosmc to support folding/activity of T-synthase. Taken together, the results demonstrate that Cosmc is an ER localized molecular chaperone required for expression of T-synthase. The systemic biochemical properties, the role of Cosmc in autoimmune diseases such as IgA Nephropathy, Tn syndrome and Henoch-Schönlein Purpura, its function in cancer biology, as well as its role in vivo are under investigation.

(135) **Determination of Membrane Topology of Yeast and Mouse Stt3p, A Highly Conserved Subunit of Oligosaccharyltransferase.**

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Stt3p is a highly conserved protein subunit of oligosaccharyltransferase(OT) in yeast and mammals. Recent evidence suggest that Stt3p may be involved in recognizing a protein substrate that carries N-glycosylation acceptor site (N-X-T/S). Interestingly, Stt3p is the subunit of OT that shares the most homology from yeast to human. The sequence alignment reveals 60% similiarity between yeast and mouse Stt3p and both proteins are predicted to have 10 to 14 transmembrane segments. In an effort to understand the structure and function of this protein, we have determined a detailed membrane topology of yeast and mouse Stt3p using insertion, truncation, and tagging of the protein in yeast and mammalian microsomes.

(136) **Expression Profiles and Transcript Stability of N-Glycan Glycosyltransferases**

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The presence of carbohydrates on cells allows the binding of several lectins, in some cases supporting cellular interactions, at other times affecting cell activity. The expression of carbohydrates can be modulated at different levels. Many factors such as tissue-specific and developmental factors have been implicated in the regulation of this wide and heterogeneous family of enzymes through specific promoter regions. Furthermore, the presence of long 5' and 3' untranslated regions (UTRs), which are involved in post-transcriptional regulation, seems to be relatively common amongst glycosyltransferases.
A sensitive method for the detection of low expressed transcripts, as is the case for glycosyltransferases, based on quantitative real-time PCR, was used to examine the expression levels and half-life times of a broad panel of glycosyltransferase mRNAs in human primary endothelial cells.
Our data indicate that high expression profiles correlate with "house-keeping" function, as in the case of the enzymes implicated in the synthesis of core N-glycans, like Golgi mannosidases and MGATs. The galactosyl- and fucosyltransferases are much less highly expressed in endothelial cells.
Within the subfamily of galactosyltransferases remarkable differences exist in the level at which the expression of their genes is regulated. Regulation at the post-transcriptional level was determined to play an important role in modulating the expression of β4Gal-T1. The presence of 4 AUUUA (AU) sequences, well known to be crucial in regulating cytokine gene expression, in the 3' UTR of its mRNA, seems to be responsible for the effect on mRNA stability. In 5 out of 7 tested members of the β4-galactosyltransferase subfamily, 2 out of 4 tested members of the β3-galactosyltransferase subfamily, and 3 out of 3 tested members of the ST3 galactosyltransferase subfamily, the 3' UTRs of their mRNAs also contain various numbers of AU sequences. It remains to be established whether these AU sequences are indeed involved in regulating the expression of the enzymes. In contrast, the expression of ST6Gal-I was determined to be solely regulated at the transcriptional level. In accordance with this observation is the lack of an AU sequence in the long 3' UTR of its mRNA. Similarly, ST6Gal-II mRNA contains no AU sequences either.
In conclusion, the expression of glycosyltransferase genes seems to be complicated and regulated at divers levels, although there might exist a common regulatory mechanism within its subfamilies.

(137) **The Characterization of Post Translational Modifications, with Special Emphasis on O-glycosylation, of Transcription Factors in Tumor Cells Variants Which Differ in their Malignant and Metastatic Capacity.**

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Structural post translational modifications of proteins by addition of a single O-linked N-acetylglucoseamine (O-GlcNAc) residues were initially described in 1984 by Carmen-Rosa Torres and Gerald W. Hart in murine Intact Lymphocytes, initially this modification was especially characteristic to proteins located at the cell surface, a more intensive investigation of this subject revealed that most of the proteins undergoing this modification are intracellular and restricted almost exclusively to the cytoplasm and nucleus. This modification is characterized by its dynamic and specific enzymatic machinery for the attachment (O-GlcNAc-transferase) or removal (â-D-Nacetylglucoseaminidase) of â linked O-GlcNAc to/from the hydroxyl groups of Ser and Thr residues, sites that can also be the same or adjacent to sites subject to modification by phosphorylation, therefore this modification seems to play a major role in structural regulation in a reciprocal manner to phosphorylation, and thus could modulate many major biological processes i.e. metabolism and signal transduction. Numerous investigation demonstrated the ubiquitous nature of structural modification by O-glycosylation, which seems to occur in all eukaryotes including animals and plants. This modification effects various protein complexes and individual proteins such as the cytoskeleton, nuclear pores, heat shock proteins and components of the transcription machinery including transcription factors. In our laboratory we investigate the T-10 fibro sarcoma (IC9 and IE7) clones, these clones express different sets of MHC glycoproteins and differ in their malignant and metastatic potential. The main goal of our current investigation was to examine the possible existence of differences in post translational modifications by O-glycosylation of several major transcription factors, in order to better understand the origin of differences in progression and signal transduction between the variant tumor cell lines, and correlate these differences to their malignant phenotype. Previous results obtained in our laboratory demonstrated the existence of profound differences in transcriptional processes in IC9 and IE7 variants of the T-10 fibro sarcoma. Preliminary results obtained by investigation these cells using EMSA and super shift assays and appropriate antibodies supported by immunoblotting and Immunoprecipitation revealed the existence of differences in O- glycosylation of NFkB and AP-1 transcription complexes. Therefore Our aforementioned results raise the possibility that post translational modifications of transcription factors by O-glycosylation may play a determinative role in the determination of tumor progression and dissemination.

(138) **Binding of Tamoxifen and its Metabolites to Alpha-1-Acid Glycoprotein Occurs Only at Supra-Physiological Plasma Concentrations of Drug**

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Background – Alpha-1-acid glycoprotein (AGP), one of the acute phase proteins, demonstrates an increase in its plasma concentration of two- to five-fold as a result of the acute phase reaction induced by inflammation and disease states. Although the precise biological function of AGP has yet to be determined, the glycoprotein exhibits high affinity, low capacity binding of basic drugs. Through binding to AGP, the free (and active) concentration of many drugs can be decreased in the plasma, which can lead to decreased efficacy. Resistance has been reported to the breast cancer therapy, tamoxifen. Suggested modalities include high levels of AIB1 protein found in breast tumour cells, altered pharmacology of tamoxifen, expression of genes that assist the growth of tumour cells and changes in the structure and function of the oestrogen receptor. Tamoxifen is primarily bound to albumin, however we sought to determine the interaction of tamoxifen citrate and its biologically active metabolites with AGP, since such binding could lead to resistance through a decrease in the free, active concentration of drug in the plasma.
Methods – The binding between drug and AGP was determined using a sensitive spectrofluorimeteric technique, based on the intrinsic fluorescence (300-400nm emission, 280nm excitation) of the tryptophan residues in the backbone of AGP. Upon binding of a drug to AGP, the peak intrinsic fluorescence is decreased (quenched), due to masking of the tryptophan residues by the drug. High quenching values correlate to a high degree of binding between drug and protein. AGP (0.75mg/mL) was tested for binding to tamoxifen citrate, 4-hydroxytamoxifen, *N*-desmethyltamoxifen, *N*-desdimethyltamoxifen, *cis*-α- and *trans*-α-hydroxytamoxifen over the range

0.1-50μM.
Results – Tamoxifen citrate at 50μM bound AGP with a quenching value of 59.4 ± 2.9%, however at the plasma concentration of ~1μM the quenching value was 4.4 ± 6.2%. 4-Hydroxytamoxifen (50μM) binding to AGP had a quenching value of 68.5 ± 2.3%; binding of 100nM drug (5x plasma concentration) was 0.1 ± 0.4%. The quenching value for *N*-desmethyltamoxifen at 50μM was 50.2 ± 0.5%; the maximal concentration in the plasma is 2.5μM and quenching was –0.5 ± 5.1% for 1μM drug. Binding of AGP to *N*-desdimethyltamoxifen at 50μM was 60.0 ± 0.5%, while for 0.5μM (peak plasma concentration is 0.26μM) the quenching was –3.9 ± 2.1%. *Cis*-α- and *trans*-α-hydroxytamoxifen (50μM) had quenching values of 39.3 ± 5.1% and 35.6 ± 3.5% respectively. There was no data to be found on the peak plasma concentration of these metabolites, however quenching values dropped to –1.3 ± 0.1% and 1.09 ± 7.1% for 0.5μM drug.
Conclusions – The binding of tamoxifen citrate and its metabolites to AGP has been confirmed using spectrofluorimetry. However the binding was observed at concentrations much greater than those found in the plasma. For tamoxifen citrate and the metabolites studied, AGP did not show a significant interaction at the peak plasma concentration for the drugs. It is therefore unlikely that AGP affects the efficacy of tamoxifen citrate and its metabolites.

(139) **Investigating Protein Clearance Mechanisms that Recognize Specific Hemostatic Components**

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One of the first identified functions for protein glycosylation was in marking circulating glycoproteins for clearance by cellular lectin receptors. Those studies indicated that exogenous and recombinant proteins show reduced half-life upon chemical and enzymatic removal of sialic acids, and increased half-life upon increased sialylation. Underlying galactose exposure, in the presence of other structural features, constitutes ligands for various lectins including the mammalian hepatic asialoglycoprotein receptor. The actual lectin-ligand relationships have remained undefined however. Recently studies have reported the role of one or more asialoglycoprotein receptors involved in clearance of von Willebrand factor in genetic models of glycosyltransferase alterations. Our previous studies of mice bearing distinct sialyltransferase lesions identified the gene encoding ST3Gal-IV as a dominant modifier of hemostasis by concealing asialoglycoprotein receptor (ASGPR) ligands. ST3Gal-IV deficient mice display a bleeding disorder associated with an autosomal dominant reduction in plasma von Willebrand factor (VWF) and an autosomal recessive thrombocytopenia. Using quantitative lectin binding analyses, increased exposure of terminal β-linked galactose was observed on plasma derived VWF and upon platelet cell surfaces. Rescue of the hemostatic defects was possible by asialofetuin administration, thereby identifying an asialoglycoprotein receptor system involved in VWF clearance. Ongoing studies include genetic complementation assays for VWF and platelet hemostasis involving mouse models of lectin deficiencies in the absence of ST3Gal-IV. The previously identified cohort of human patients with low VWF levels and high galactose exposure ratios were analyzed for mutations in the ST3Gal-IV, ST3Gal-VI and ST6Gal-I sialyltransferases. Results of these sequencing studies will be presented.

(140) **Endogenously Produced Ganglioside GM3 Endows Anti-Cancer Drug Resistance Phenotype by Upregulating Bcl-2 Expression in Lung Carcinoma Cells**

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Gangliosides constitute a class of sphingolipids which mainly expressed on cell membranes and the relationship between gangliosides and cancer malignancy has long been focused because the ganglioside patterns have dramatically changed during cell differentiation and cancer malignancy (Hakomori, 1989, Hakomori, 1994, Hakomori and Igarashi, 1995). Especially, ganglioside GM3, a major ganglioside in many mammalian cells, was found to be abundant in highly metastatic lines of B16 melanoma (Yogeeswaran et al., 1978, Nozue et al., 1988). The cisplatin-resistant small-cell lung cancer cell line, SBC-3/CDDP, showed a marked increase in ganglioside GM3 compared with the parent SBC-3 cells (Kiura et al., 1998). And more, transfection of cytosolic sialidase cDNA into a highly metastatic and invasive cell line, B16-BL6, exhibited markedly decreased experimental pulmonary metastasis (Tokuyama et al, 1997). On the other hand, transfection of the plasma memblane-associated sialidase gene into human colon cancer cells inhibited apoptosis and accompanied by increased Bcl-2 and decreased caspase-3 expression (Kakugawa et al., 2002). And murine bladder carcinoma cell line, MBT-2, transfected with sialyltransferase-I (SAT-I) cDNA was characterized by a reduced potential for invasion, xenograft tumor growth and an increase in the number of apoptotic cells (Watanabe et al., 2002). These conflicting findings surrounding ganglioside GM3 and malignancy may be not only the influence of ganglioside GM3 but also the change of the total ganglioside composition may involve. So far, little is known concerning the mechanisms through which specific gangliosides induce malignancy. Thus, to investigate the significance of ganglioside GM3 in cancer malignancy, we established ganglioside GM3-reconstituted cells by transfecting cDNA of GM3 synthase into the J5 subclone of 3LL Lewis lung carcinoma cells. The J5 subclone was selected for the transfection of this gene because it lacks GM3 but accumulates lactosylceramide. DNA fragmentation induced by TNFf_{i} of both GM3-reconstituted cells and mock-transfected cells were similar. However, the GM3-reconstituted cells was resistant to apoptosis induced by etoposide and adriamycin. Because of non-specific caspase inhibitor suppressed these DNA fragmentation, we investigated the activation of caspases by these apoptosis inducers, and found that caspase-3 and -9 weren't activated by etoposide in the GM3-reconstituted cells. Moreover, the level of Bcl-2 protein was increased in the GM3-reconstituted cells. There was no difference between the GM3-reconstituted cells and mock-transfected cells in the expression of P-glycoprotein and incorporation of adriamycin. Wild type 3LL Lewis lung carcinoma cells which expresses GM3 rich didn't show DNA fragmentation induced by etoposide and expressed higher level of Bcl-2 protein compared to the GM3-defficient J5 subclone. Thus, these results support that endogenously produced GM3 positively involves malignant phenotypes including anti-cancer drug resistance probably through upregulating Bcl-2 protein.

(141) **Production and Phenotypic Analysis of Mice Lacking the *Mgat4a* and *Mgat4b* Gene-Encoded Glycosyltransferases GlcNAcT-IVa and GlcNAcT-IVb**

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We are investigating the in vivo functions of specific N-glycan branch patterns using the intact mouse as a genetic model system. Mammalian N-glycan branching can include N-acetylglucosamine linked to the α3-linked core mannose of N-glycans, thereby initiating formation of a distinct branch structure found on some hybrid and complex N-glycans. We have designed targeting constructs that use Cre-loxP recombination and have carried out gene-targeting of the *Mgat4a* and *Mgat4b* alleles in embryonic stem cells to enable tissue-specific mutagnesis and germline gene deletion in vivo. The *Mgat4a* gene is abundantly expressed in small intestine, large intestine, and pancreas. Systematic inactivation of the *Mgat4a* gene resulted in a significant deficiency of GlcNAcT-IV activity in these and other tissues. Homozygous *Mgat4a*-null mice were found to be viable and reproduced normally. Hematological and serum chemistry analyses did not reveal any significant differences between *Mgat4a*-null mice and their littermate controls. Histologic analyses using datura stramonium lectin nevertheless revealed alterations in N-glycan structure in the absence of *Mgat4a*. Other findings among mice lacking *Mgat4a* will be presented. Mice lacking *Mgat4b* are currently under initial characterization. The potential need to study mice lacking both *Mgat4a* and *Mgat4b* may be indicated to adequately determine the function of this distinct N-glycan branch structure.

(142) **Effect of TNFalpha and TGFbeta1 on the Glycosylation of Bovine Synoviocytes**

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Inflammatory cytokines, such as those present in rheumatoid arthritis, have been shown to alter the sugar chains of glycoproteins, by altering the expression and activities of glycosyltransferases and sulfotransferases. In this study, we examined the relationship between the biosynthesis of glycoprotein-

bound sugar chains and growth and apoptosis of bovine synoviocytes in response to the presence of cytokines TNFa and TGFb1. Bovine synoviocytes (synovial lining fibroblast-like cells) were isolated from calf synovial tissue, and grown in culture with and without TNFa or TGFb. Cell numbers were assessed to determine growth. Apoptosis was examined by annexin V and TUNEL staining by fluorescence microscopy as well as by flow cytometry. To characterize cell surface glycosylation, cells were stained with biotin-labeled lectins in enzyme-linked lectin binding (ELLA) assays. Glycosyltransferase and sulfotransferase activities involved in glycoprotein biosynthesis were measured using specific enzyme assays. Bovine synoviocytes were found to have activities of the enzymes synthesizing simple O-glycan structures with core 1 (Gal b1-3 GalNAc), as well as GlcNAc-transferases I and II synthesizing branches of complex N-glycans. Enzymes involved in chain elongation and termination, such as Gal-, sulfo-, sialyl- and Fuc-transferase activities were also detected. TNFa treatment did not change cell numbers but induced apoptosis in synoviocytes. In addition, lectin binding assays showed that TNFa induced significant increases in terminal GlcNAc and sialic acid residues, in O-glycan core 1, and in N-glycan structures. This was associated with decreased activities of the b3-Gal-transferase that synthesizes core 1, and decreased activities of a2-Fuc-transferase and core 1 sulfotransferase. In contrast, activities of core 2 b6-GlcNAc-transferase and b4-Gal-transferase synthesizing the branched core 2 O-glycans were increased. The results suggest that apoptotic synoviocytes have a tendency to form more complex O-glycan structures. The effect of TGFb on glycosylation appeared to be different from that of TNFa. A comparison with previous work suggests that the effects of cytokines on cellular glycosylation are cell type and cytokine-specific. Supported by The Arthritis Society and the Canadian Cystic Fibrosis Foundation.

(143) **Core 2 *ƒ*À-1,6-N-Acetylglucosaminyltransferase (C2GnT) Expression in Human Prostate Cancer: A Predictor for Non-Organ Confined Disease and Biochemical Relapse after Radical Prostatectomy**

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Purpose: Core 2 *ƒ*À1,6-N-acetylglucosaminyltransferase (C2GnT) is a key enzyme to form core 2 branched O-glycans whose expression in colorectal cancer and pulmonary adenocarcinoma has been associated with advancement of the disease. It has been demonstrated that C2GnT expression of colorectal cancer cells and pulmonary adenocarcinoma cells is positively correlated with progression of the disease. However, there are no attempts to elucidate the role of C2GnT expression in prostate cancer. The subject of this study was to test whether immunohistochemical detection of C2GnT in biopsy specimen can predict pathological stage and prostate specific antigen (PSA) relapse in patients with organ confined prostate cancer treated with radical prostatectomy.

Materials & Methods: The prostate needle biopsy specimens of 69 men treated with radical prostatectomy were immunohistochemically examined for C2GnT status. Uni- and multivariate analyses were carried out to compare C2GnT status to other well-known preoperative variables (PSA, clinical stage, biopsy Gleason score (GS) and percent of positive cores for cancer) to determine which variable independently predicted pathological stage and PSA relapse.

Results: Univariate analysis showed that PSA, biopsy GS and C2GnT status were significant predictors of pT3 disease (p = 0.006, 0.046 and 0.002, respectively). PSA and C2GnT status were significant predictors for PSA relapse (p = 0.001 and 0.011, respectively). Multivariate analysis revealed that percent of positive cores and C2GnT status were independent predictors of pT3 disease (p = 0.016 and 0.006, respectively). PSA and C2GnT status were independent predictors for PSA relapse (p = 0.004 and 0.028, respectively).

Conclusions: Preoperative immunohistochemical detection of C2GnT is an independent predictor of non-organ confined disease and PSA relapse following radical prostatectomy.

(144) **Sulfation as Tuning**

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Sulfated glycans, especially of mucin type, are known to be lectin ligands. Sulfation of glycan can increase the interaction affinity and recognition specificity as documented for interaction of L-selectin with its ligand. At the example of several lectins we have systematically tested the effect of glycan sulfation on affinity and interaction specificity of an interaction. To do this we have synthesized a set of sulfated oligosaccharides bearing Su group at position 3 or 6 of galactose unit or position 6 of hexosamine residue: core disaccharides of type 1, 2 and 3, and more complex oligosaccharides with additional fucose and/or sialic acid residues.

Siglecs of sialoadhesin family did not interact with OS where sialic acid was replaced to Su residue. Additional Su at position 6 of sialo-OS hexosamine usually did not affect the interaction with the exception of the pair siglec-xx/Neu5Aca2-3Galb1-…(6-Su)GxxNAc when the interaction was completely abolished due to the sulfate presence.

The example of opposite kind is the interaction of human galectin-1 with Galb1-3GalNAca: non-substituted disaccharide of type 3 bound galectin very weakly whereas the sulfated disaccharide interacted with K_d comparable to that of LacNAc.

The third example is interaction of H5N1 avian influenza viruses (Hong Kong outbreak, 1997) with sialoreceptor. Introduction of additional sulfate in position 6 of trisaccharide Neu5Aca2-3Galb1-4GlcNAc but not to the corresponding type 1 and 3trisaccharides increased interaction with viral hemagglutinin ~10 times.

Thus, sulfation of the glycan allows either to increase or to decrease lectin-carbohydrate interaction, acting not only by switching *on/off* principle, but also in tuning mode.

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(145) **Identification of Candidate Glycoproteins Involved in Human Airway Epithelial Wound Repair *In Vitro***

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Asthma is a disease of the airways described as reversible airflow obstruction. Asthmatics have an accelerated decline in lung function over time compared to healthy subjects. One of the phenotypic changes associated with the pathology of asthma is chronic damage to the epithelium. Repair of airway epithelium after injury involves the spreading and migration of cells to re-establish the physical barrier followed by the proliferation and differentiation of cells to restore tissue integrity. Glycoproteins are functionally important in cell signaling, adhesion, migration and proliferation. Differences in the surface glycosylations between normal cells to those in the wound margin have been shown using lectin cytochemistry. Our laboratory has demonstrated *Allomyrina dichotoma* lectin (AlloA) is a lectin that differentially stains epithelial cells in damaged areas of the epithelium compared to the staining of intact epithelium. We *hypothesize* that AlloA could be used as a marker to identify functional glycoproteins involved in the epithelial repair of wounded airways. Monolayer wound repair was examined with $1HAE_0$- (human airway epithelial cells) in culture as the model. Small circular wounds (~ 1 mm^2) were made on monolayers of $1HAE_0$- cells growing on coated and uncoated plates and stained them with different lectins by lectin cytochemistry. Considering that AlloA may identify relevant glycoprotein(s) involved in repair, we treated the wounded monolayers with AlloA (10-100 µg/ml) and EGF (15 ng/ml) and followed wound closure over 24 hrs. In a wounded monolayer, AlloA stained cells that migrate towards and into the wound with the accumulation of positive staining cells in the wounded area over time. Human EGF stimulated wound closure; however, wound closure was inhibited in a dose-dependant manner when cells were co-treated with AlloA. Inhibition did not appear to be due to a loss in cell viability. Identification of the proteins AlloA binds was carried out by a modified lectin-precipitation procedure. Biotinylated AlloA was used to precipitate proteins with the carbohydrate structure recognized by AlloA. Avidin-agarose beads were used to co-precipitate proteins now bound by the AlloA lectin. Following gel electrophoresis of the AlloA precipitated proteins, tryptic digestion and MALDI-TOF mass spectrometry was performed on relevant protein bands and peptide mapping utilized for identification. We have successfully identified candidate glycoproteins from peptide sequencing.

Future work will include determining the expression of the candidate glycoproteins and/or their receptors in normal and in asthmatic airways as well as looking at the glycosylation structures to determine sequence and structure as well as function and their relationship in asthma.

(146) **Golgi α1,2-Mannosidase IB Null Mice Display Neonatal Respiratory Distress Caused by Pulmonary Hemorrhage**

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Golgi α1,2-mannosidases form the $Man_5GlcNAc_2$ precursor required for hybrid and complex *N*-glycan biosynthesis. Golgi α1,2-mannosidase IB is one of three mammalian paralogs (IA, IB, IC) that have similar specificities. Northern blots indicate that the paralogs display distinct developmental and tissue-specific expression. The physiological role of Golgi α1,2-mannosidase IB was investigated by gene ablation in the mouse using the Cre/loxP recombination system. Null fetuses display no gross differences and develop to term. However, null allele neonates display symptoms of respiratory distress and perish within hours of birth. Histological analysis revealed pulmonary hemorrhage similar to that observed in some preterm infants suffering from respiratory distress. Alterations in *N*-glycan biosynthesis are being investigated using lectins and immunohistochemical analysis is underway to characterize the etiology of the respiratory distress. Supported by the Canadian Institute of Health Research (CIHR).

(147) **Apolipoprotein Glycosylation and Its Potential Influence in Atherogenesis**

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Apolipoproteins play important roles in plasma lipid transport, cellular lipid flux and, in specific cases, exhibit functions in cell biology that are apparently not related to lipid transport. A major focus of apolipoprotein research has been to understand their role in pathways that modulate atherogenesis. At least 21 apolipoproteins occur in humans. Of these, 9 are known glycoproteins and 17 are predicted to be glycosylated based on sequence homology analysis for O-glycosylation or the presence of the N-X-S/T sequon for N-glycosylation. The amount of carbohydrate present in apolipoproteins varies from 1 glycan per polypeptide (eg. apoE) to accounting for up to 25 to 30% of the apolipoprotein mass (eg. apo(a) and apoJ, respectively). As is the case for most glycoproteins, the analysis of apolipoprotein glycan structure and function is in its infancy. Here we report on the glycosylation of two plasma apolipoproteins, apoB100 and apo(a), and the novel insights provided in our understanding of their potential roles in atherogenesis. ApoB100 is the sole protein component of low density lipoprotein (LDL), the most abundant cholesterol transport lipoprotein in plasma. Previous studies, using non-specific methods, indicated that a reduced sialic acid content of LDL contributed to its atherogenicity. It was hypothesised that desialylation of apoB100 N-glycans increased its propensity to accumulate in arteries and thereby promote atherosclerosis. Indeed, treatment of LDL with sialidase increased its pro-atherogenic properties in vitro. Furthermore, sub-populations of small dense LDL (sdLDL), which are associated with atherosclerosis risk, were found to be sialic acid deficient and assumed to represent a desialylated LDL fraction. We used exoglycosidase sequencing and mass spectrometry to analyse fluorescently labelled apoB100 glycans. The glycans were N-linked and largely present (93%) as complex biantennary and oligomannose structures. Eighteen glycans were characterised in total and the glycosylation pattern was found to be consistent in apoB100 derived from different donors, including those with increased atherosclerosis risk (i.e. Type 2 diabetes and familial hypercholesterolaemia) and in all LDL sub-populations in both control and hypercholesterolaemic subjects. Using similar methodology we analysed LDL glycolipid composition and showed that sdLDL was relatively deficient in GM3 thereby providing a novel explanation for the previous assertion than sdLDL represents a desialylated LDL fraction. Apo(a) occurs in plasma linked to apoB100 via a single disulphide bond. The apo(a)-apoB100 complex forms a lipoprotein termed Lp(a). Apo(a) and its proteolytic fragments are pro-atherogenic but the factors that regulate the specific apo(a) fragmentation pattern found in vivo are unknown. We elucidated apo(a) glycan structures and found that they were mostly (83%) O-linked core type 1 structures. The N-glycans were mono- and di-sialylated complex biantennary structures. We provide evidence that the absence of O-glycans at specific interkringle linker domains of the apo(a) protein increase its susceptibility to proteolytic fragmentation thus providing an explanation for the specific apo(a) fragmentation pattern found in vivo. These data demonstrate the potential importance of the analysis of apolipoprotein glycosylation in order to understand how apolipoproteins contribute to atherogenesis.

(148) **Description of Four Common Polymorphisms in CDG-I Related Human Genes *MPDU1*, *ALG12* and *ALG2***

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Congenital Disorder of Glycosylation (CDG) are autosomal recessive disorders characterized by central nervous system dysfunction and multisystemic disorders associated with defective serum glycoprotein N-glycosylation. CDG-I comprises ten distinct defects in the assembly of the lipid-linked oligosaccharide chain and its transfer to protein, noted from CDG-Ia to CDG-Ij. Untyped cases are labelled CDG-x until they are fully characterised. In order to diagnose CDG-x non-Ia, -Ib and –Ie, which have no enzyme analysis facilities, we screened by sequencing analysis all the CDG-I related genes already described in our French patients. Amplification of CDG-I related genes were performed on genomic DNA and sequencing analysis was performed on an Abi Prism 3100 (Applied Biosystem). We found four subtitutions : G685A (A229T) on *MPDU1* (CDG-If), A1177G (I393V) on *ALG12*, (CDG-Ig), T31C (S11P) and C1100T (V367A) on *ALG2* (CDG-Ii) at the heterozygous state in three patients, one patient had the two substitutions in the *ALG2* gene. A229 in *MPDU1* and I393 in *ALG12*, are amino acids conserved between species but S11 and V367 in ALG2 are not conserved from human to *S. cerevisiae*. We screened by restriction analysis the four substitutions in 50 to 55 unrelated healthy French individuals. A1177G (I393V), C1100T (V367A) and T31C (S11P) creating a Hinc II, a Hha I or abolishing a Hinf I restriction site respectively. We developed a diagnostic method of RFLP assay with mutagenic primer introducing a diagnostic HhaI restriction site for the G685A (A229T) substitution. The PCR products were digested and then electrophoresed in a 3% agarose or a 12% bis-acrylamide gel. All four substitutions were found to be common polymorphisms with frequency of 16%, 8.2%, 10 and 7.5% for G685A (A229T), A1177G (I393V), T31C (S11P) and C1100T (V367A) respectively. These polymorphisms may interfere with other mutation detections and thus should be taken into account. Furthermore, as a common polymorphism F304S in ALG6 gene (CDG-Ic) was found to exacerbate the clinical severity of patients with CDG-Ia, it could be the same with some of these polymorphisms.

(149) **Leukocytes Facilitate Metastasis through L-Selectin Mediated Interactions**

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Hematogeneous metastasis of carcinomas is accompanied by aggregation of platelets and leukocytes forming tumor cell emboli. It was shown that primarily P-selectin mediates platelet-tumor cells interactions, which are critical for metastasis. Recently, we have shown that also L-selectin is involved in metastasis (PNAS 99: 2193-2198), where in L-selectin deficient mice attenuation of metastasis was observed. We now show that L-selectin mediates the interaction of granulocytes and monocytes with the tumor cell emboli, and that heparin also inhibits L-selectin mediated interactions, thereby metastasis. Here we provide evidence that the initial clearance of intravenously injected tumor cells is not different between wild type (L-sel+/+) and L-selectin deficient mice (L-sel-/-). However, the number of surviving tumor cells is higher in the L-sel+/+ mice after 24 h and later. Based on the staining for presence of platelets and fibrin, the size and the composition of the tumor emboli was evaluated and found comparable in both mouse types. A detailed analysis of the mouse lung section revealed a temporal difference in the number of leukocytes associated with the tumor emboli. Immunofluorescence analysis with several leukocyte markers revealed a marked difference in the amount of leukocytes present in tumor emboli. Neutrophils and monocytes in L-sel+/+ mice were almost entirely absent in L-sel-/- mice. The presence of leukocytes in tumor cell emboli was detected already 12 h post-tumor cell injection and sustained for another 24h. In L-sel-/- mice, no association of leukocytes was detected. The role of L-selectin in mediation of neutrophils and/or monocytes association with tumor cells was studied in vivo. The intravenous injection of L-selectin blocking antibody reduced metastasis indicating its involvement in this process. Previously, we reported that heparin given prior to tumor cell injection can effectively inhibit metastatic

progression mainly due to inhibition of P-selectin mediated platelet-tumor cell interactions. However, heparin is also an effective ligand for L-selectin. Here we show that tumor cell injection, followed by injection of heparin several hours later, led to attenuation of metastasis. The time-point of heparin injection was chosen so that no interference with P-selectin inhibition was expected. Thus, heparin inhibits also L-selectin-dependent interaction leading to attenuation of metastasis. In this regard, it is of clinical relevance that heparin treatment leads to an attenuation of metastasis even when the tumor cells are already in circulation, reflecting the common situation in patients. These results provide further insights into the mechanism of L-selectin action and suggest that leukocytes can facilitate metastasis and that heparin is effective in preventing them.

(150) **Analysis of Stored Oligosaccharides in a Mouse Model of a Glycolipid Storage (Sandhoff) Disease**

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Sandhoff disease is an inherited disorder in which GM2 ganglioside and related glycolipids are stored in the lysosome, due to genetic defects in the lysosomal enzymes, β-hexosaminidase A and B, that are required for the catabolism of GM2 and related glycolipids. The brain is mainly affected in this disease and patients usually die by 2-4 years of age. Currently, non-invasive means of monitoring disease progression in the brain of individuals with Sandhoff disease are not available. The aim of this study was to identify biochemical and/or magnetic resonance (MR) visible markers of disease progression in a knockout mouse model of Sandhoff disease.

Brains from Sandhoff mice (n= 14) at four different age points (presymptomatic to terminal stage) and age matched wild-type C57Bl/6J mice (n=9) were extracted with perchloric acid (PCA). The PCA extracted brain samples were analysed by 1-dimensional (^{1}H) and 2-dimensional (^{1}H-^{1}H gCOSY and ^{1}H-^{13}C gHMQC) magnetic resonance spectroscopy (MRS). These extracts were further analysed for the presence of free oligosaccharides, following fluorescent labelling with anthranilic acid (2-AA), using HPLC and MALDI-TOF mass spectrometry. For magic angle spinning MRS (MAS-MRS) experiments, brain slices were prepared from a separate group of Sandhoff (n=6) and wild-type (n=3) mice. Brains were sliced (1-2 mm) and analysed by solid state MAS-MRS.

In vitro metabolite profiling by MRS identified a 'new' resonance at δ 2.07 ppm in the Sandhoff mouse brain proton spectrum, which correlated to a ^{13}C resonance at δ 23.4 ppm. These chemical shifts are characteristic of the presence of an N-acetyl group. This is to be expected if storage of PCA-extractable, free oligosaccharides containing non-reducing terminal N-acetylglucosamine (GlcNAc) residues, which are the substrate for β-hexosaminidase, occur in Sandhoff mice similar to that reported in Sandoff patients[1]. This peak was present in all the Sandhoff mice and in none of the wild-type mice. HPLC and mass spectroscopy confirmed the presence of free oligosaccharides with the composition of $Hex_{(2-3)}HexNAc_{(2-5)}$ in the PCA extracts. The presence of GlcNAc was confirmed following digestion with β-hexosaminidase. Furthermore, the predominant oligosaccharide species $Hex_3HexNAc_4$ (52.8 %) is similar in structure and composition to a $Man_3GlcNAc_4$ oligosaccharide, which is the primary stored oligosaccharide (70%) in the brains of Sandoff patients[1]. Analysis of intact brain tissues by MAS-MRS also showed that this metabolite peak appears to increase from the presymptomatic to terminal stage of disease. This increase is likely due to both the accumulation of free oligosaccharides and the increasing storage of GM2 ganglioside and related glycolipids in Sandhoff mouse brains.

These experiments demonstrate the presence of a new metabolite peak in the Sandhoff mouse brain MRS spectra. This peak is apparently due to N-acetyl groups on HexNAc residues. Since, in the *ex vivo* experiments performed by MAS-MRS, this peak appeared to correlate with disease progression, it may be a viable marker for monitoring disease progression and response to experimental therapies.

Reference:

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(151) **Heterologous Expression of Galectin-3 in Murine Melanoma Decreases Cell Growth and Sensitizes to Induced Cell Death In Vitro.**

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Systematic analysis of proteins expressed in melanoma cells had contributed to the identification of genes associated with melanoma progression. Based on combined approaches both in human and murine models, we had observed a decrease of galectin-3 accumulation in vertical growth phase melanoma cell lines. Here we have used a murine melanoma variant (TM1 cells), cloned in our laboratory, to evaluate the role of galectin-3 in the malignant phenotype. TM1 cells are amelanotic, express the murine melanoma antigen, bear normal TP53 alleles and express neither CDKN2a (p16) nor galectin-3. TM1 cells were transfected with the human galectin-3 gene inserted in pEF1-neo plasmid (kindly provided by Dr. Fu-Tong Liu, UCDavis). Stable transfectants of either TM1.neo or TM1.neo/gal-3 cells were cloned and analyzed regarding cell growth and death. Galectin-3 expression led to a consistent delay in TM1 doubling time in vitro in all clones analyzed. Cell cycle analysis of TM1.neo/gal-3 cells suggested an accumulation of cells in G0/G1 phases. No spontaneous apoptosis was observed upon galectin-3 expression. However, when cells were exposed to staurosporin, galectin-3 expressing cells were more susceptible to cell death. Therefore, expression of human galectin-3 in murine melanoma cells led to a negative modulation of cell proliferation and a positive modulation of cell death. These somehow unexpected results suggest that either (1) galectin-3 functions may vary within the different cellular contexts; or (2) galectin-3 intracellular functions depend on molecular interactions involving non-conserved domains of galectin-3, such as the proline-rich region. In this latter case, human galectin-3 could act as dominant negative in murine cells. These results call for the dissection of the molecular interactions of galectin-3 that may explain its multiple functions within different intracellular compartments. Supported by FAPESP.

(152) **Basis for Acquisition of PNA High Phenotype in Activated Peripheral CD8+ T Cells**

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Lymphocyte development and activation have been associated with changes in glycosylation of cell surface glycoproteins and glycolipids. Documented changes include terminal glycosylation of glycoprotein glycans such as Sialyl-Le^x, the expression of specific glycolipids (GM1, asialo-GM1) and changes in Core 1 and Core 2 O-glycans. In thymus, immature medullary thymocytes bind the plant lectin PNA, which recognizes the O-linked glycan Galβ1,3GalNAcαThr/Ser (Core 1). In contrast, mature thymocytes in the cortex are PNA low due to sialylation of Core 1 by ST3Gal I (1, 2). Several reports have documented an increase in PNA binding to activated peripheral lymphocytes, accounting for the use of PNA as a marker of germinal centers in spleen and lymph nodes. We are investigating the molecular basis for the increased expression of PNA receptors following activation of peripheral CD8+ T cells. The binding of PNA, analyzed by flow cytometry occurs in virtually all activated CD8+ T cells within 72 h of *in vitro* activation. Glycoproteins carrying PNA receptors were analyzed by PNA agarose precipitation followed by PNA blot. This revealed that CD45 is the major PNA binding glycoprotein in activated CD8+ T cells. Interestingly, the expression of the glycolipid asialo-GM1, which contains the terminal Galβ1,3GalNAc sequence recognized by PNA, was also increased in activated CD8+ T cells. However, a similar increase in PNA binding was observed in activated CD8+ T cells from a GM2/GD2 synthetase null mouse strain, which does not express complex gangliosides including asialo-GM1. Our results showed that asialo-GM1 may contribute to the PNA binding, but is not a major carrier of PNA receptors on activated CD8+ T cells. The contribution of changes in glycosyltransferases and/or sialidases to the increase in PNA binding to activated CD8+ T cells is being evaluated using various approaches including real time PCR and enzyme assays. Results to date suggest that increased expression of PNA receptors does not involve sialidases, but can be accounted for by decreased expression of the sialyltransferase ST3Gal I. (Supported by NIH grant AI050143)

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(153) **Down-Regulation of Tumor-Associated N-glycans in GnT-V Deficient (GnT-V$^{-/-}$) Mice**

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Many tumor cells show specific changes in cell surface glycan expression during oncogenesis and metastasis; a frequent change is the increase in N-linked β (1,6) branches caused by up-regulation of the transcription of a glycosyltransferase, N-acetylglucosaminyltransferase V (GnT-V). Increased expression of GnT-V has been reported in different tumor tissues and correlated with metastatic potential of tumors. Deletion of GnT-V (GnT-V$^{-/-}$) suppressed tumor growth and metastasis [1]. Here, we showed the alterations in N-glycan in GnT-V knock-out mice using lectin precipitation and HPLC analysis of fluorescently labelled oligosaccharides after N-glycanase (PNGase F) treatment, as well as possible mechanisms involved in inhibition of tumor growth and metastasis in GnT-V deficient mice. Decreased N-linked β (1,6) branches and poly-N-acetyllactosamine were observed in all tissues in GnT-V deficient mice. In some tissues, the formation of α 2,3 sialylation and fucosylation structure were also inhibited in GnT-V deficient mice, but little change was observed in formation of α 2,6 sialylation. Consistent with decreased expression of α 2,3 sialylation and fucosylation, the sialyl-Lewis^x carbohydate epitope was decreased significantly in some tissues from GnT-V deficient mice. Moreover, embryo fibroblasts from GnT-V deficient mice showed phenotype changes associated with decreased tumorigenesis and metastasis including decreased cell growth, survival, and focal adhesion turnover, consistent with the down-regulation of tumor-related N-glycans. These observations suggest that deletion of GnT-V caused decreased formation of some tumor-related N-glycans, which may contribute to the inhibition of tumor growth and metastasis.

Key words: GnT-V deficient mice, N-linked glycan, tumor-related phenotype

[1] Granovsky M et al. 2000 *Nat Med* **6**, 306-312

(154) **Regulation of Cytokine Receptor Residency and Signaling by N-glycosylation**

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Glycoform variation on T cell receptors regulates their affinity for galectin-3, and thereby antigen-dependent receptor clustering and susceptibility to autoimmune disease in vivo (Demetriou et. al. Nature, 409:733, 2001). Cytokine signaling is dependent on receptor trafficking through membrane microdomains, and glycosylation may also control their distribution in a similar manner. We have found that modification of the Asn (N)-linked glycans on cytokine receptors by UDP-N-acetylglucosamine:a-6-D-mannoside -β1,6 N-acetylglucosaminyltransferase V (Mgat5) lowers the response threshold to multiple cytokines, including TGF-β, EGF, PDGF and IGF cytokines. Mgat5-modified N-glycans bind galectin-3 forming a multivalent glycoprotein lattice that increases the residency-time of N-glycosylated receptors at the cell surface. The glycoprotein-galectin lattice reduced loss of surface EGF and TGF-β receptors into early endosomes and caveolae. The signaling deficiency in Mgat5-deficient cells impaired the invasive phenotype of tumor cells, and extravasation rate of macrophage in vivo. An Mgat5-deficient tumor cell line depleted of caveolin-1 (Cav-1) displayed rescue of cytokine signalling, but not the glycoprotein-galectin lattice. (Cav-1), an intra-membrane protein, localizes to cholesterol-enriched microdomains and sequesters multiple signaling proteins. Our results suggest that Cav-1 and the cholesterol-microdomains may “buffer” cytokine signaling, while the galectin-glycoprotein lattice promotes switch-like kinetics in the EGF response. Polylactosamine is highly expressed during embryogenesis, when switch-like signaling kinetics is probably required during morphogenesis. Cav-1 protein levels increase with age, and may contribute to the suppression of acute cytokine signaling, while sustaining basal signaling in tissues. Rescue of the Mgat5 defect in galectin-3 EGFR interaction, as well as signaling was accomplished by supplementing the growth medium with GlcNAc or GlcN. Glucose flux through the hexosamine pathway to UDP-GlcNAc promoted poly N-acetyllactosamine synthesis and Mgat5-modifications of cell surface N-glycans on cytokine receptors. Our results indicate that the lattice provides positive feedback between glucose supply and receptor signaling, which stimulates glycolysis and cell growth. Cytokine receptors that induce anabolic growth display a higher density of N-glycans than other families of glycoprotein receptors, consistent with a role in receptor by the glycoprotein-galectin lattice. Finally, Mgat5-deficient mice display evidence of metabolic insufficiency with an age-related loss of lean tissue mass, reduced blood glucose, and early mortality. Supported by grants from CIHR and US Army.

(155) **A Link between Protein-Losing Enteropathy and Loss of Heparan Sulfate Proteoglycans**

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Protein-losing enteropathy (PLE), the loss of plasma proteins through the intestine can exceed 20 g/day. It occurs in seemingly unrelated diseases, e.g. Crohn's disease, Congenital Disorders of Glycosylation (CDG), or after surgery to correct congenital heart malformations. These diverse diseases share several common features: PLE is episodic and heparan sulfate proteoglycans (HSPG) on the basolateral surface of intestinal epithelial cells are absent or mislocalized during these episodes. The importance of HSPGs for PLE is supported by prior evidence that the electrostatic charge provided by the highly anionic HSPGs prevents albumin loss through the glomerulus in patients with nephrotic syndrome. In addition, episodic PLE onset is often associated with a pro-inflammatory state, suggesting multiple factors combine to exceed critical thresholds. However, mechanisms that link PLE with a loss of HSPG and an inflammatory response are yet unknown. Our objective was to investigate the importance of HSPG as a factor for the development of PLE. Combinations of loss of heparan sulfate (HS) and the exposure to the pro-inflammatory cytokine TNF-α were studied. An in vitro model of PLE was established using a monolayer of intestinal epithelial cells (HT29) in transwells, and protein leakage was analyzed using FITC-conjugated albumin. In parallel, transepithelial electrical resistance (TEER) was measured to monitor monolayer integrity. Heparanase treatment or TNF-α treatment significantly increased the paracellular albumin flux and decreased TEER in a concentration dependent manner. Soluble HS blocked heparanase-mediated increase in albumin flux, while chondroitin sulfate did not. The effect of TNF-α was more pronounced than that caused by heparanase. The effects of combining heparanase and TNF-α were additive, suggesting different pathways may be involved. In conclusion, removal of HS chains from the basolateral surface of intestinal epithelial cells increases pericellular albumin flux and the effect is potentiated by TNF-α. Our findings support the notion that PLE can result from multiple independent or interdependent processes, some of which may involve HSPG. (Supported by the Children's Hearts Fund)

(156) **Regulation of *N*-Glycosylation and Cytokine Signaling by the Hexamine Pathway**

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Defects in glucose sensing are associated with obesity, type II diabetes, life span, as well as the lifetime risk of cancer. In single cell eukaryotes, glucose is the ligand that stimulates G protein-coupled receptors linked to Ras-adenylate cyclase, and an Akt-related signaling pathway to control metabolism, and critical cell size. In metazoans, similar intracellular signaling pathways are down-stream of a large family of morphogenic cytokines and receptor kinases that trigger developmental processes, in addition to glucose uptake and glycolysis. Mgat5$^{-/-}$ tumor cells are defective in the glycoproteins-galectin lattice controlling cytokine receptor residency at the cell surface and growth signaling (Partridge, Dennis). The mutant cells are smaller, with reduced glycolysis and display decrease PI3 kinase/Akt activation. Glucose flux through the hexosamine pathway to UDP-GlcNAc promotes poly *N*-acetyllactosamine synthesis and Mgat5-modifications of cell surface *N*-glycans on cytokine receptors. Glucose-6-P is converted to fuctose-6-P, a key intermediate supplying glycolysis, the pentose phosphate pathway and the hexosamine pathway. Glutamine:fructose-6-P aminotransferase (GFAT), transfers the amino group from glutamine and isomerization of fructose-6-P to yield GlcN-6-P, an irreversible reaction in mammalian cells. Transgenic over-expression of GFAT, or chronic administration of GlcN or uridine and in rodents elevates UDP-HexNAc and causes insulin resistance. However, the effectors down-stream of the glucose and the hexosamamine pathway are not known. Recent evidence suggests *N*-glycosylation of cytokine receptors may regulate their association with lectins at the cell surface, and thereby signaling thresholds (Partridge, Dennis).

To assess the effects of the hexosamine pathway on *N*-glycans biosynthesis and structures Mgat5$^{+/+}$ and Mgat5$^{-/-}$ cell lines were grown in DMEM medium with and without 40 mM GlcNAc supplement. The GlcNAc or GlcN supplements increased intracellular pools of UDP-GlcNAc by ~5 fold in Mgat5$^{+/+}$ cells and ~20 fold in Mgat5$^{-/-}$ cells relative to Mgat5$^{+/+}$ grown in DMEM. Low molecular weight N-linked glycan profiles from the cells were generated on a MALDI-CFR mass spectrometer. The concentration of branched glycans in Mgat5$^{-/-}$ cells grown in GlcNAc-supplemented medium was higher than in Mgat5$^{-/-}$ in normal DMEM and lower than in Mgat5$^{+/+}$. GlcNAc supplemented cells showed a larger variety of complex-type and lower abundance of high mannose *N*-glycans. Furthermore, high molecular weight N-linked glycans were increased in abundance by the GlcNAc supplement cells for both Mgat5$^{-/-}$ and Mgat5$^{+/+}$ cell lines. Supplements of GlcN and GlcNAc appeared to be enhanced for poly *N*-acetyllactosamine synthesis and Mgat5-modifications. Our structural analysis indicates that the hexosamine pathway can regulate branched and polylactosamine content of glycoproteins. Therefore the galectins-glycoprotein lattice may provide positive feedback between cytokine signaling pathways and glucose metabolism. Supported by BRIN-NCRR (VR), NIGMS (VR), CIHR (JD), and US Army (JD).

(157) Disruption of Murine Mannose Phosphate Isomerase (*Mpi*) Produces Embryonic Lethality through An Apparent Energy Deprivation Mechanism

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Phosphomannose isomerase (PMI; EC 5.3.1.8) catalyzes the interconversion of fructose-6-phosphate and mannose-6-phosphate (Man-6-P), biochemically linking glycolysis to glycan synthesis. PMI deficiency in humans leads to incomplete glycosylation and causes the Congenital Disorder of Glycosylation Type Ib (CDG-Ib). Ten mutations in human PMI are currently known that produce CDG-Ib syndrome. However, no patients with a complete deficiency of PMI have been identified. Deficient glycosylation and aberrant phenotypes are completely corrected with a mannose-supplemented diet. In contrast, mannose is highly toxic to honeybees, depleting their ATP as a result of high hexokinase and very low PMI activity that restricts Man-6-P flux into glycolysis. This is known as the honeybee effect. Utilizing a retroviral-based promoter trap strategy in embryonic stem cells, we have disrupted the murine mannose phosphate isomerase (Mpi) gene that encodes PMI. Heterozygous Mpi mice appear completely normal. In contrast, Mpi null mice are embryonic lethal, surviving only to 11.5-dpc. A noticeable intra-uterine growth retardation (IUGR) develops in homozygotes at E9.5 and continues to 11.5-dpc with increased severity and 17.4% null recovery. Placental hyperplasia associated with null embryos is evident at E11.5, although preliminary data suggests no functional deficiency of trans-placental nutrient transport. Mannose supplementation accelerates the embryonic lethality of null embryos, with none surviving to 11.5-dpc. Furthermore, in-vitro labeling of embryonic fibroblasts prepared from null embryos with 3H-mannose reveals a substantial intracellular build-up of Man-6-P. Together, these data imply that excessive mannose causes the embryonic lethality due to a honeybee effect that depletes ATP and produces energy starvation. However, it remains to be determined whether normal levels of maternal serum mannose are alone sufficient to cause the embryonic lethality or if insufficient protein glycosylation also plays a role. (Supported by RO1065091)

(158) Kinetic Analysis of Phosphoglucomutase Using A Novel Mass Spectrometry based Strategy

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Phosphoglucomutase (PGM) catalyzes the interconversion between glucose-1-phosphate (Glc1P) and glucose-6-phosphate (Glc6P), and plays an important role in the synthesis and breakdown of glycogen. Conventionally, kinetic measurement of PGM is performed through the use of a coupling enzyme and spectrophotometric detection. Due to the possible interference of the coupling enzymes with the kinetic properties of the enzyme of interest, a direct assay for this isomeric substrate-product pair is desirable. Recently, our laboratory has developed a method to quantify isomeric monosaccharides phosphorylated at either the 1- or the 6- position by using gas phase ion/molecule reactions and FT-ICR mass spectrometry[1]. Using the diagnostic ions in their corresponding ion/molecule reaction spectra and a multicomponent quantification method, the composition of isomeric binary mixtures has been accurately determined. In the work presented herein, this methodology is further applied to the kinetic measurements of PGM.

For K_M and V_{max} measurements of the substrate Glc1P, the enzymatic reaction was quenched with ACN at certain time intervals and then run through a strong canion exchange column to remove Mg^{2+}. The product concentration was calculated by multiplying the total concentration of the two isomers (the initial substrate concentration) and the product percentage determined through the above quantification methodology. A product-time curve was first generated to determine the optimum reaction time, and 3 min was chosen in this case. The initial velocity was thus determined by dividing the product concentration by the reaction time. Based on the saturation plot for Glc1P, average values of K_M and V_{max} were determined to be 12.89 μM and 148.53 μmol/mg/min, respectively. Due to the unfavorable K_{eq} for this reaction, product-time plot for the other substrate Glc6P departs from linearity at low substrate conversion percentage. Therefore it is difficult to measure K_M for Glc6P using the saturation plots. As reported in previous literatures[2], K_M for Glc6P is calculated using the Haldane relationship: $K_{eq}=[P]_{eq}/[S]_{eq}=(V_{max}^f/K_{M,S})/(V_{max}^r/K_{M,P})$. To measure K_{eq}, PGM was added to three solutions containing pure Glc1p, pure Glc6p or a 1:1 mixture of Glc1P and Glc6P as starting substrates. After overnight reaction, these mixtures were analyzed to determine the ratio of [Glc6P] to [Glc1P] at equilibrium. K_{eq} was calculated to be 17.8 with these three measurements. V_{max}^r was measured using 1mM Glc6P as the starting substrate. At 3% substrate conversion, V_{max}^r was calculated to be 50 μmol/mg/min. According to the Haldane relationship, K_M for Glc6P was determined to be 74.63 μM. All these kinetic constants agree well with reported values [2]. Current research is focused on inhibition studies of several nucleotide inhibitors. This strategy is also promising in kinetic measurements of other members of the phosphomutase class.

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(159) Comparison of the Kinetic Properties between Two GnT-IV Isozymes.

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N-acetylglucosaminyltransferase-IV (GnT-IV) catalyzes the formation of GlcNAcβ1-4 branch on GlcNAcβ1-2Manα1-3 arm of the core structure of *N*-glycans, which is an essential in producing multiantennary structure cooperatively GnT-V. To date, two human GnT-IV genes named IVa and IVb had been identified. The sequence identity between them was 62 % in amino acid sequence. The IVa mRNA was expressed in a tissue specific manner, while the expression of the IVb mRNA was constitutive. In this study, to elucidate the roles of GnT-IV isozymes in *N*-glycan biosynthesis, we compared enzymatic properties of two isozymes. Soluble truncated forms of human GnT-IVa and -IVb containing a *N*-terminal FLAG tag were generated and expressed in HFK293T cell. The proteins were purified by anti-FLAG antibody gel affinity chromatography and their substrate specificities were determined. Both GnT-IV showed similar substrate specificities toward tested fifteen PA- derived acceptor sugar chains. The tri-antennary sugar chain having GnT-V product was the best acceptor for GnT-IVa and -IVb. Both enzymes had a relatively low activity toward the hybrid type *N*-glycan (Man_5Gn), which was about 25 % of the activity with bi-antennary complex type *N*-glycan substrate. Both GnT-IV could act on GlcNAcβ1-2Manα1-3Manβ1-4$GlcNAc_2$-PA. However, GlcNAcβ1-2Manα1-6Manβ1-4$GlcNAc_2$-PA was not a substrate, indicating that the importance of the tri-saccharide structure (GlcNAcβ1-2Manα1-3Manβ1-) for substrate recognition by GnT-IV. While K_m values for UDP-GlcNAc of both GnT-IV were almost same, K_m value for bi-antennary sugar chain of IVb was 7-times higher than that of IVa. Moreover V_{max} of IVb was 1.6-times lower than that of IVa. The differences in these kinetic parameters mean that IVa is more active enzyme than IVb and it contributes branch formation more than GnT-IVb under the physiological condition.

(160) **The Michaelis Constants Ratio for Two Substrates with Mold and Yeast β-Galactosidases**

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A comparative analysis of kinetic data for fungal β-galactosidases has revealed that the ratio of Michaelis constants with two substrates (lactose and NPG) is approximately constant for certain groups of enzymes. MCR = K_M(lactose) / K_M(NPG) equals 35 ± 3 and 10 ± 1.5 for the mold and the yeast enzymes respectively. Thus, MCR can be used for the identification of related enzymes. MCR for a group of enzymes with a pair of standard substrates may serve as an identification number of this group. Additional information is needed for evaluation of its potential correlation with the classification of glycoside hydrolases based on amino acid sequence similarities. A deviation of MCR for a particular enzyme from the group parameter might be an indication of possible errors in K_M determination, or in assignment of the enzyme to this group. Within the framework of classical Michaelis-Menten kinetics and in accordance with the customary use of K_M for a comparison of enzyme-substrate affinity, the MCR parameter may be interpreted as a relative substrate affinity. This proportion remains nearly the same for different mold (or yeast) β-galactosidases, though the affinity to a particular substrate may vary essentially from enzyme to enzyme. The constancy of MCR may be a manifestation of structural similarity of binding sites for these enzymes.

(161) **Identification of A Low-Affinity Mannose 6-Phosphate Binding Site in Domain 5 of the Cation-Independent Mannose 6-Phosphate Receptor**

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Cation-independent mannose 6-phosphate receptor (CI-MPR) and cation-dependent mannose 6-phosphate receptor (CD-MPR) are type I membrane glycoproteins that function in the targeting of lysosomal enzymes to lysosomes. The extracytoplasmic domain of the 300 kDa CI-MPR has 15 repeating units and the two high-affinity carbohydrate binding sites have been mapped to domains 1-3 and 9. Domain 5 of the CI-MPR exhibits the highest sequence homology to domain 3 and domain 9 than any of the other domains of the receptor. Sequence alignment prediction indicates that domain 5 contains the four key primary amino acid determinants identified as essential for carbohydrate recognition by the CD-MPR and domains 3 and 9 of the CI-MPR. However, it lacks the two cysteines corresponding to Cys106 and Cys141 in the CD-MPR sequence which are involved in a critical disulphide bond forming the mannose 6-phosphate (Man-6-P) binding pocket. Therefore, to test the hypothesis that domain 5 harbors a low-affinity Man-6-P binding site, a construct encoding domain 5 alone was expressed in *Pichia pastoris* system with a C-terminal His-tag. The truncated receptor (Dom5His), purified by Ni-NTA metal affinity chromatography, revealed multiple 17-20 kDa species corresponding to the various unglycosylated and glycosylated forms of the protein. Enzymatic deglycosylation and mass spectrometric analyses identified that the two potential *N*-glycosylation sites are utilized adding an *N*-glycan mass up to ~3000 Da. Chemical cross-linking of Dom5His revealed dimeric and higher oligomeric forms in addition to the monomeric species. Oligosaccharide-based microarray analysis determined that the purified Dom5His recognized pentamannosyl 6-phosphate. There was no binding to high-mannose *N*-glycans. These results were corroborated by preliminary equilibrium binding data which indicated a binding affinity (K_d) of >1 μM for the lysosomal enzyme, *β*-glucuronidase.
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(162) **Mutation Met344His in Bovine β1,4-Galactosyltransferase-1 Broadens Its Primary Metal Ion Specificity**

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β1,4-Galactosyltransferase (β4Gal-T1) transfers galactose from UDP-galactose (UDP-Gal) to N-acetylglucosamine (GlcNAc) in the presence of a metal ion. A transition metal ion, and not an alkaline earth metal ion such as magnesium, is required to activate the enzyme. Recent crystallographic studies on the bovine β4Gal-T1 have shown that the primary metal binding site is located at the hinge region of a long flexible loop that undergoes a conformational change during the catalytic cycle of the enzyme. Furthermore, the binding of Mn^{2+} at this site involves an uncommon coordination to the Sδ atom of Met344 residue. To test whether this coordination is responsible for the requirement of a transition metal ion, Met344 residue was mutated to His or Glu. These mutants exhibit enzymatic activity in the presence of a wide range of metal ions, including alkaline earth metals, which do not activate the wild-type enzyme. In the presence of Mg^{2+}, the mutant, M344H-Gal-T1, exhibits 25% of the catalytic activity observed with the wild-type enzyme in the presence of Mn^{2+}. It also has higher K_m for the substrates. The crystal structures of M344H-Gal-T1 in complex with either UDP-Gal.Mn^{2+} or UDP-Gal.Mg^{2+}, and the crystal structure of M344E-Gal-T1 in complex with UDP-Gal.Mn^{2+}, have been determined at 2.3 Å resolutions. The structures show that the coordination stereochemistry of Mg^{2+} is quite similar to that of Mn^{2+}. Both His344 and Glu344 in the mutants exhibit stronger coordination bonds with the metal ion compared to Met344 in the wild-type enzyme. This strong metal-ion coordination in the mutants, it is concluded, results in reduced k_{cat} by not only interfering with the ability of the long flexible loop to undergo the required conformational changes during the catalytic cycle but also by interfering with the formation of the transition state complex. Funded in part by DHHS #NO1-CO-12400.

(163) **Metformin-Stimulated Mannose Transport In Dermal Fibroblasts**

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The biguanide drug metformin is widely used to treat Type II diabetes. It has antihyperglycemic activity due to two effects: attenuation of gluconeogenesis in hepatocytes, and stimulation of glucose transport by skeletal muscle. Metformin reportedly acts by stimulating AMP-activated protein kinase (AMPK), a master regulator of cellular energy metabolism. We have identified a metformin-stimulated D-mannose transport (MSMT) activity in human dermal fibroblasts. The EC_{50} for MSMT (0.3 - 0.5 mM) was 10-fold lower than that for basal D-mannose transport (3 - 5 mM). MSMT was specific for the 3-, 4-, and 6-OH groups of the D-mannose ring, but not the 2-OH group, and thus could be inhibited by D-glucose and 2-deoxy-D-glucose. Yet, MSMT was not readily explained by greater glucose transporter activity because metformin did not increase transport of 2-deoxy-D-glucose or 3-O-methyl-D-glucose by fibroblasts. MSMT was significant: in the presence of only 2.5 micromolar mannose, MSMT corrected experimentally induced deficiencies in both the synthesis of glucose$_3$mannose$_9$GlcNAc$_2$-P-P-dolichol and the modification of nascent proteins with N-linked high-mannose glycans. MSMT was also identified in Congenital Disorder Of Glycosylation Type Ia and Ib fibroblasts, and we are currently evaluating the ability of metformin to correct the glycosylation defect in Ia cells. In conclusion, it appears that activation of AMPK in fibroblasts with metformin results in the expression of a mannose-selective transport system. Supported by NIH grant GM38545 and Welch Foundation grant I-1168

(164) **Is Glutamate 317 (E317) of α-1,3-Galactosyltransferase Important for Enzyme Activity?**

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The enzyme α-1,3-Galactosyltransferase (α-1,3GT) catalyzes the transfer of galactose from the donor substrate, uridine diphosphate-α-galactose (UDP-α-Gal), to the galactose residue of the N-acetyllactosamine (Gal-β-1,4-GlcNAc-R) acceptor substrate. This reaction uses Mn^{+2} as co-substrate to produce the product Gal-α-1,3-Gal-β-1,4-GlcNac. The crystal structure (2.3 Angstroms resolution) of this enzyme, initially reported by Gastinel et al. in 2001, demonstrates that the enzyme contains ten β-strands, six α-helixes, and six 3_{10} helixes. Furthermore, the structure suggest that a highly conserved residue, glutamic acid 317 (E317), is positioned to act as the catalytic nucleophile involved in transfer of galactose with retention of the stereochemistry at C1 of the sugar.

In the present study, site directed mutagenesis was used to substitute either alanine (E317A), aspartic acid (E317D), cysteine (E317C), or histidine (E317H) for E317 to determine if this amino acid is critical for enzyme activity. It was predicted that conservative mutations would result in enzymes that retained at least some activity, whereas non-conservative mutations would generate inactive enzymes. As anticipated, the conservative mutant E317D retains activity, albeit significantly less than the wild type enzyme. Mutants containing less conservative substitutions (i.e. E317C and E317H) also had low activity. An unexpected result was that the non-conservative mutant

E317A also retained enzyme activity, suggesting that E317 is not the catalytic nucleophile proposed in the double inversion mechanism. Therefore, a different amino acid may act as the catalytic nucleophile or the reaction may proceed by a different mechanism.

(165) **N-Glycan Patterns of Human Serum in Rheumatoid Arthritis**

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Rheumatoid arthritis (RA) is considered to be an autoimmune disease characterized by destructive polyarthritis. Immunoglobulin G (IgG) is a glycoprotein with biantennary complex-type N-glycans attached to Asn297. There have been a lot of reports about N-glycan alternation in IgG of RA patients(1), and these glycan structures seem to be closely correlated to the disease progression. However, the current analysis of IgG N-glycans requires many tedious procedures including a purification of IgG in serum. This process is considerably limiting an application of the N-glycan analysis to the clinical diagnosis of RA. An aim of this study is to simplify the RA N-glycan analysis procedures by eliminating the IgG purification for a practical diagnostic method of the RA N-glycans.

N-glycans were released from glycoproteins by means of N-glycosidase F digestion. Reducing ends of the released N-glycans were derivatized with 2-aminopyridine(PA)(2) and then, PA N-glycans were separated by ODS and Amide columns, monitored using a fluorescence detector, and characterized by the so-called two-dimensional HPLC mapping technique(3).

We analyzed 18 kinds of neutral N-glycans obtained from serum. The result showed that relative amounts of agalactosyl biantennary complex-type N-glycans (Fuc +, bisecting GlcNAc -) are remarkably higher than those of monogalactosyl ones, and there is a significant correlation with relative amounts of N-glycans between the serum and the purified IgG which N-glycan alternations were well known. This result suggests that the serum N-glycan analysis may be an easier diagnostic method of RA without a time/cost consuming. In addition, LC/MS based method will be presented.

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(166) **Purification and Crystallization of Protein Disulfide Isomerase, an Enzyme that Catalyzes the Folding of Glycoproteins.**

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The formation and rearrangement of disulfide bonds in newly synthesized glycoproteins is catalyzed by protein disulfide isomerase (PDI), an enzyme in the endoplasmic reticulum. It has also been reported that PDI has chaperone activity for folding of N-linked glycoprotein and can function as a subunit of other protein complexes such as prolyl-4-hydroxylase[1]. PDI is a glycoprotein but it has been proved that glycosylation is not essential for it full activity. The primary sequence of PDI can be divided into 4 domains: a-b-b'-a' and an acidic C-terminal tail. In both the a and a' domains, there is an active site motif -CGHC-. However, how these active sites function is still not well understood. An atomic structure of PDI will help clarify the catalytic mechanism. The single domain structures of the a and b domains have been resolved by NMR for mammalian PDI[2, 3], and both domains were shown to consist of a thioredoxin fold. However, until the structure of full-length PDI is determined, it is still unclear how the active sites are spatially oriented and how they carry out the catalytic process. We have overexpressed the full-length yeast PDI in *E. coli*. The protein was purified sequentially by affinity, anion-exchange chromatography and gel filtration. Crystallization was attempted with several crystal screen kits, and crystals of yeast PDI was obtained. Diffraction data was collected at the National Synchrotron Light Source at Brookhaven National Laboratory. On the basis of a 4.0Å dataset, the space group of PDI crystal was assigned as $P2_1$ with unit-cell dimensions: a=84.9Å, b=75.7Å, c=84.9Å, α=90.0°, β=93.0°, γ=90.0°. Currently, we are optimizing the crystallization conditions and exploring the heavy metal replacement method to solve the 3D structure of PDI. (Supported by NIH Grants GM33184 to WJL and DK54835 to HS)

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(167) **Identifying the Elements Involved in Glycoprotein Recognition by Peptide:N-Glycanase**

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Newly synthesized N-linked glycoproteins are folded in the endoplasmic reticulum (ER) and enter the secretory pathway, while the misfolded glycoproteins are retained in the ER and subsequently exported to the cytoplasm, where they are subjected to proteasomal mediated ER associated degradation (ERAD). Cytoplasmic Peptide:N-Glycanase (Png1p) contributes to glycoprotein degradation by catalyzing the hydrolysis of the β-aspartylglycosylamine bond of asparagine-linked glycopeptides and glycoproteins. Png1p has been shown to deglycosylate a fetuin-derived asialoglycopeptide (1) and it was recently reported that Png1p and its mammalian counterpart could deglycosylate full-length glycoproteins (2). Deglycosylated cytoplasmic intermediates have also been observed in cells treated with proteasome inhibitors, suggesting that Png1p acts before the proteasome. However, it has not been firmly established if Png1p deglycosylates the misfolded glycoproteins before proteasomal degradation or whether it acts after glycoprotein degradation.

To establish the deglycosylation pathway of Png1p and to identify the elements responsible in the glycoprotein-Png1p interaction, we chose yeast carboxypeptidase Y (CPY) as a model substrate. Our results showed that yeast Png1p could completely deglycosylate heat denatured CPY, while the native glycoprotein remained intact. As expected, the catalytic triad mutants of Png1p were unable to deglycosylate misfolded CPY. To explore the possibility that the N-glycan chain is involved in the glycoprotein-Png1p interaction, we performed a co-immunoprecipitation of denatured CPY with Png1p. Contrary to expectations, the denatured, deglycosylated CPY remained bound to Png1p, suggesting the possibility that Png1p recognizes the peptide backbone in addition to the β-aspartylglycosylamine bond. We propose that this interaction may be mediated by the N-glycosylation motif: N-X-S/T on the glycoprotein (or glycopeptide). To test this hypothesis first we intend to study the binding of pNGase F (bacterial)-deglycosylated CPY with Png1p. Bovine serum albumin (BSA) will be used as a control to rule out non-specific binding of Png1p to denatured protein. Finally, to identify the key residues involved in the interaction, a peptide-binding assay of Png1p with Ac N-X-S/T amide, and Ac N-X-A amide as a negative control will be performed. Thus, the proposed experiments would help in our understanding of recognition and deglycosylation of glycoproteins by Png1p (Supported by NIH Grant GM 33184).

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(168) **Autoglucosylation Activity of Proteoglycogen**

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We have described the purification of rabbit muscle proteoglycogen. Mild digestion of the purified proteoglycogen with α-amylase led us to prepare PG-200, a proteoglycogen species having a polysaccharide moiety of lower size than the native species (Carrizo, M.E., Miozzo, M.C., Goldraij, A., and Curtino, J.A. (1997) Glycobiology 7, 571-578). The polysaccharide bound glycogenin of PG-200 showed increased activation energy and lower catalytic activity for transglucosylation of DBM than polysaccharide-free glycogenin (Romero, J.M., Carrizo, M.E., Montich, G. and Curtino, J.A. (2001) Biochem. Biophys. Res. Commun., 289, 69-74). We now describe the ability of PG-200 for autoglucosylation when incubated with UDP-14C-glucose. During the incubation some polysaccharide-free glycogenin was released form PG-200 due to a slight contamination of the PG-200 preparation with α-amylase. However, polysaccharide-free glycogenin proved not to be responsible for the

14C-glucosylation of PG-200. After inactivation of samples by heating, the incubations with UDP-14C-glucose of mixtures of inactive PG-200 with active polysaccharide-free glycogenin and inactive free-glycogenin with active PG-200 resulted in no glucosylation. This indicated the inability of any of both glycogenin species to serve as acceptor for the other. A residual autoglucosylation activity was also observed in the native proteoglycogen fraction isolated from rabbit muscle. The apparent discrepancy between the autoglucosylation of polysaccharide-bound glycogenin and the cessation of polysaccharide-free glycogenin autoglucosylation when the acquired oligosaccharide moiety reached 8-11 glucose units, is discussed. Supported by FONCYT, CONICET, SECyT-UNC and Agencia Cordoba Ciencia grants.

(169) **Isolation and Characterization of An Insect Gene Encoding A β1,4-N-Acetylgalactosaminyltransferase Involved in LacDiNAc Biosynthesis.**

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While the terminal portions of many glycoprotein glycans contain N-acetyllactosamine (LacNAc; Galβ4-GlcNAc-R), some, particularly in invertebrates, contain N,N'-diacetyllactosdiamine (LacDiNAc; GalNAcβ4-GlcNAc-R). This disaccharide is produced when a β1,4-N-acetylgalactosaminyltransferase transfers N-acetylgalactosamine in β1,4-linkage to a terminal N-acetylglucosamine residue. Previously, van Die and coworkers demonstrated that lepidopteran insect cells, including a cell line derived from *Trichoplusia ni*, have β1,4-N-acetylgalactosaminyltransferase activity (Glycobiology 6:157). This result was interesting because it strengthened a growing body of evidence that insect glycoprotein glycan processing pathways might be more extensive than generally recognized and that insect cells might be able to produce complex N-glycans. As part of our long-term effort to elucidate glycoprotein processing pathways in insect systems, we have molecularly cloned a β1,4-N-acetylgalactosaminyltransferase cDNA from *Trichoplusia ni*. Bioinformatic analyses indicated that this cDNA (Tnß4GalNAcT) belongs to the β1,4-galactosyltransferase family. However, biochemical analyses demonstrated that the Tnß4GalNAc-T gene product transfers N-acetylgalactosamine from UDP-GalNAc, not galactose from UDP-Gal, to a p-nitrophenyl N-acetyl-β-D-glucosaminide acceptor. Additional biochemical and cell biological analyses indicated that the Tnß4GalNAc-T gene product is a β1,4-N-acetylgalactosaminyltransferase. Furthermore, we found that this gene product can transfer N-acetylgalactosamine to glycoprotein glycans in vivo. These results indicate that the cDNA isolated in this study encodes a β1,4-N-acetylgalactosaminyltransferase involved in the production of LacDiNAc during N-glycoprotein biosynthesis in insect cells. This enzyme is closely related to the *C. elegans* enzyme (Ceß4GalNAcT) recently shown to be involved in LacDiNAc biosynthesis by Kawar and coworkers (J. Biol. Chem. 277:34924). However, there are also some interesting differences between the insect and worm β1,4-N-acetylgalactosaminyltransferases, which will be detailed in the poster.

(170) **Rational Design of β1,4-Galactosyltransferase-I with High Glucosyltransferase Activity**

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β1,4-Galactosyltransferase-I (Gal-T1) normally transfers Gal from UDP-Gal to GlcNAc in the presence of Mn^{2+} ion (Gal-T activity). In the presence of α-lactalbumin (LA), the Gal acceptor specificity is altered from GlcNAc to Glc. The enzyme does not have an absolute requirement for the sugar donor UDP-Gal; rather, it exhibits polymorphic donor specificity, in that it also transfers glucose (Glc), d-deoxy-Glc, arabinose, GalNAc, and GlcNAc from their UDP derivatives, albeit at low rates (0.3-5%) compared to Gal transfer. Earlier we have shown that LA enhances the transfer of Glc from UDP-Glc to GlcNAc by the wild-type enzyme (Glc-T activity) from 0.3 to 8% of its Gal-T activity. The crystal structure of the Gal-T1.LA.UDP-Glc complex reveals that LA does not interact with the sugar nucleotide. The low Glc-T activity exhibited by Gal-T1 may be due to the hydrogen bonding interaction between O4 hydroxyl group of Glc moiety and the side chain carboxylate group of Glu317. Since mutation of Glu317 reduces the Gal-T1 activity, we considered mutating the residues in the vicinity of the Glu317 to enhance the Glc-T activity in the absence of LA. Crystal structure also revealed that the side chain guanidine group of Arg228 forms a hydrogen bond with the side chain carboxylate group of Glu317. Therefore, the Arg228 residue was considered for the mutational studies. Furthermore, the side chain of Arg228 forms the base of the catalytic pocket, where its positively charged guanidine group is placed between the side chain carboxylate group of Asp252 and Glu317 residues. In order to keep the positive charge between these two negatively charged side chain carboxylate groups, and still form a hydrogen bond with the side chain of Glu317, Arg228 was replaced by Lysine. As expected, the single mutant R228K exhibits enhanced Glc-T activity compared to wild-type Gal-T1, and LA further enhances this activity nearly 6 fold. The enzyme kinetic studies reveal that the k_{cat} for the Glc-T activity of the mutant in the absence and the presence of LA, is nearly 40 and 56 fold higher than the wild type respectively. The mutant exhibits reduced Gal-T activity compared to the wild type. Although the K_m values for the substrates have not changed in the Gal-T activity, it exhibits a dissociation constant (K_{ia} = 243 μM) for the UDP-Gal molecule. In the UDP-Gal bound crystal structure of the mutant, Asp318 residues was found to have two possible side chain orientations; one orientation corresponds to the wild type which is away from the Gal moiety of UDP-Gal, whereas in the alternate orientation Asp318 forms a short contact with the Gal moiety causing a steric hindrance. Thus presence of such a steric hindrance by Asp318 for Gal moiety may have been responsible for the observed dissociation of UDP-Gal from the mutant enzyme (K_{ia} = 243 μM). Further details on the UDP-Glc bound structure and its relevance to enzyme kinetics will be presented. Funded under Contract No. NO1-CO-12400.

(171) **Mammalian-like *O*-Linked Motifs on Plant Glycoproteins**

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Most of the plant glycobiology studies have been carried out on the biochemistry and molecular biology of *N*-linked glycosylation. However, there is very little information available on mammalian-like *O*-glycans in plants. Mammalian mucin-type *O*-linked glycan structure on rice glutelin was first reported in 1999 (Kishimoto et al.). To study the potential *O*-glycosylation in plants, we further examined rice proteins. We found that rice prolamines carried Galβ1-3GalNAc motif which is the core type I in mammalian *O*-linked glycans. These studies suggest that mammalian-type glycan moieties are present on plant endogenous proteins. Ref; Kishimoto et al., Arch. Biochem. Biophys. 370, 271-277 (1999)

(172) **Structure and Dynamics of a Cell Surface Mucin Motifs**

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Mucin glycoprotein domains are important components of the cell surface landscape, particularly in immune cells. Molecular interactions with such domains in normal and diseased cells have been implicated in a variety of cellular and inter-cellular responses. In an effort to better understand the molecular recognition events in which these participate, we have used NMR methods to develop a high resolution structural description of mucin segments by examining well-defined chemically synthesized mucin glycopeptide fragments with NMR. Initial efforts have focused on the N-terminal sequence, STTAV, from CD43. (1) Glycoconjugates carrying the Tn, TF, and STF epitopes on S1, T2 and T3 have been examined, elucidating the specific hydrogen bond and hydrophilic interactions between the first alpha-linked GalNAcs of the glycans and the peptide backbone. These brace a stable glycopeptide core scaffold, and are the basis of the special structural characteristics of these molecules. In contrast, such a situation was not found for beta-linked GalNAc residues. Even at the level of an alpha-linked glycopeptide of this size, an exceptionally stable conformation is seen, giving strong support to the contention that such a structural arrangement is highly favored energetically, and is reflective of its organization in the full glycoprotein. The core organization seems largely invariant to the size of the pendant glycan. The features are substantially maintained when an SSS glycosylated triplet is substituted for the analogous STT. Expanding on these results, the dynamics of the glycopeptide core are being investigated using C-13 NMR relaxation measurements. The structural and dynamic experimental measurements provides a basis for cross-validating with results from molecular dynamics computational models, and will therefore help in extending the ability to model more complex systems. Results from C-13 NMR relaxation measurements and molecular dynamics computations, along with additional structural studies will be presented.

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(173) **Monoglucosylated Oligosaccharide in Lepidopteran Storage Proteins**

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Arylphorin of the Chinese oak silkworm, *Antheraea pernyi* has a unique oligosaccharide structure of $Glc_1Man_9GlcNAc_2$. We investigated the generalized pattern of monoglucosylated oligosaccharide from different lepidopteran arylphorins of the Japanese oak silkworm, *Antheraea yamamai* and silkworm, *Bombyx mori*. Arylphorins were purified by gel-filtration chromatography and SDS-PAGE. All arylphorins had a peak that eluted at the same position as the $Glc_1Man_9GlcNAc_2$ of *A. pernyi*, even though they were extracted from different lepidopteran species and genus. The glycan peak was apparently digested by rat liver α-glucosidase II which is specific to α1,3Glc of $Glc_{1\text{-}2}Man_9GlcNAc_2$, and then co-eluted with $Man_9GlcNAc_2$ on normal-phase HPLC. Interestingly, Met-rich protein, which is another kind of a hexameric storage protein expressed in *B. mori*, also had the same size of glycan as standard $Glc_1Man_9GlcNAc_2$, suggesting that the monoglucosylated oligosaccharide might generally occur in lepidopteran storage proteins. The relative amount of $Glc_1Man_9GlcNAc_2$ of storage proteins, however, varied between silkworm species and showed considerable seasonal variation for *A. pernyi* larvae. Considering constant protein conformation apart from the relative variation of glycan amount, it may not be the monoglucosylated glycan structure but location for a function. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) with Con A Sepharose-eluted fraction of trypsinized *A. pernyi* arylphorin showed that oligosaccharides were present at second and third site, although the corresponding gene had four sequons for *N*-glycosylation. In addition, only the second site was occupied by the monoglucosylated oligosaccharide and a little amount of $Man_{7\text{-}9}GlcNAc_2$. It was confirmed by *N*-terminal amino acid sequencing and MALDI MS for each glycopeptide fractionated by reversed-phase HPLC. It suggested that local structure of the storage proteins or metabolic rate of a given organism might affect to produce $Glc_1Man_9GlcNAc_2$. It was likely that $Glc_1Man_9GlcNAc_2$ was generated by partial deglucosylation of $Glc_{2\text{-}3}Man_9GlcNAc_2$ not by protein re-glucosylation, since there were no detectable oligosaccharides such as $Glc_1Man_{7\text{-}8}GlcNAc_2$. It indicates that folding of lepidopteran storage proteins may be completed with partially deglucosylated oligosaccharide.

(174) **Expectation of Oligosaccharide Function in A Lepidopteran Storage Protein of the Chinese Oak Silkworm, *Antheraea pernyi***

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The full-length cDNA of *Antheraea pernyi* arylphorin containing a signal sequence was isolated by using RT-PCR and cloned into the pET 28a vector as a preparation for mutagenesis in glycosylation. Repeated nucleotide sequencing with the PCR product and cloned plasmid confirmed the cDNA sequence of arylphorin gene. The sequence information was deposited to Genbank (AY278025). The amino acid sequence of *A. pernyi* arylphorin showed 63-77% homology with reported arylphorins of lepidopteran species including *A. yamamai, Bombyx mori, Hyalophra cecropia*, and *Manduca sexta*. *A. pernyi* arylphorin contained four potential N-glycosylation sites, in which the first two sites were conserved in arylphorins; however, practical glycosylation was characterized at the second (^{196}Asn-Tyr-Ser) and third (^{348}Asn-Asp-Ser) site. A homology model structure of *A. pernyi* arylphorin was constructed by computer-aided modeling technique with reported hemocyanin crystal structure to expect the function of oligosaccharides in the storage protein. It appeared that ^{196}Asn of the second site was buried within the pocket of anti-parallel α-helix in the arylphorin, while ^{348}Asn of the third site was partially exposed at the protein surface. In addition, it seemed that ^{348}Asn was located near the center of the arylphorin trimer, and two trimers would then form a hexamer. It suggested that $Glc_1Man_9GlcNAc_2$ was an incompletely processed oligosaccharide due to restricted accessibility by ER ¥á-glucosidase II which is responsible for the removal of two α 1,3Glc from $Glc_2Man_9GlcNAc_2$ glycan of folding glycoprotein, involved in completely folded and assembled storage protein. All these results imply that oligosaccharides at the second or third site may play a role in the folding of arylphorin subunits or in the assembly of hexamer, respectively. The amino acid of ^{196}Asn or ^{198}Ser at the second site, and ^{348}Asn or ^{350}Ser at the third site were replaced to alanine by site-directed mutagenesis, respectively, and the sequences were confirmed by nucleotide sequencing. A wild type and mutants genes were expressed in *E. coli* and insect cell line, *Sf*9, and verified the changed amino acids by MALDI MS/MS. A biochemical and biophysical study will be carried out with native and the expressed storage proteins.

(175) **Oligosaccharide Ligands on Human Colon Cancer Cells Associated with an Anti-tumor Activity of Serum Mannan-Binding Protein**

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Mannan-binding protein (MBP), a Ca2+-dependent mammalian lectin specific for mannose, N-acetylglucosamine and fucose. The serum MBP activates complement through the lectin pathway. The lectin plays an important role in 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, which was called MBP-dependent cell-mediated cytotoxicity (MDCC) 1) In order to elucidate the mechanism of MDCC, we have studied the nature of the MBP-ligand oligosaccharides expressed on the human colorectal carcinoma cell line, SW 1116. FITC-MBP binding to SW1116 cells was inhibited specifically by the haptenic sugars and also by fucose-specific lectins such as AAL. The anti-Lewis b mAb inhibited the MBP-binding most effectively, followed by anti-Lewis a mAb, whereas the anti-Lewis x, anti-Lewis y and anti-sialyl Lewis a mAbs did not inhibit the binding significantly, suggesting that the MBP recognizes and binds to Lewis b and Lewis a epitopes. Thus, the lectin appears to distinguish the Type 1 and Type 2 chains but hardly distinguish Lewis a and Lewis b epitopes. From the glycopeptide fraction prepared from the pronase digest of SW1116 cell lysates, MBP-ligand oligosaccharides were released by hydrazinolysis and pyridyl-aminated and then isolated by an MBP affinity column. The PA-derivatized MBP-ligand oligosaccharides were fractionated into the neutral and acidic fractions by a DEAE column. Analyses of the carbohydrate composition and reducing end sugars of the neutral MBP ligand oligosaccharide fraction indicated that the oligosaccharides are N-glycans with high molecular size consisting mainly of galactose, N-acetylglucosamine and fucose. Endo-β-galactosidase digestion of the ligand oligosaccharides resulted in a marked reduction of the binding activity to the MBP column together with the decrease of their molecular sizes, indicating the presence of type 2 chains structure in the MBP ligands and the importance of the large molecular size as the functional MBP-ligands. The selective removal of fucose residues from the MBP-ligand oligosaccharides by TFA treatment resulted in almost complete loss of the binding activity to MBP and AAL affinity columns, confirming the vital importance of fucose residues as an epitope. A combination of MALDI-MS/MS and nano ESI-MS/MS analyses indicated the presence of the Lewis a/b structures on the non-reducing ends of the ligands. MBP-ligand oligosaccharides, which are suggested to trigger MDCC, appear to be high molecular weight poly N-acetyllactosamine-type sugar chains with Lewis a and Lewis b epitopes on their branch ends. Reference: 1) Ma, Y. et al.: Proc. Natl. Acad. Sci. USA, 96, 371-375, 1999

(176) **Selective Loss of Epimerase Activity of the UDP-N-Acetylglucosamine 2-Epimerase/N-Acetylmannosamine Kinase due to Site-directed Mutagenesis**

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N-Acetylneuraminic acid (Neu5Ac) is an essential molecule in many recognition systems. Its biosynthesis is regulated by the bifunctional enzyme, UDP-N-acetyl-glucosamine 2-epimerase/N-acetylmannosamine kinase (GNE/MNK) (1). The N-terminal region of this bifunctional enzyme displays similarities with prokaryotic UDP-GlcNAc 2-epimerases, whereas the sequence of its C-terminal region is similar to sequences of members of the sugar kinase superfamily. Mutations of the gene encoding the GNE/MNK have been implicated as the cause of sialuria, which seems to result from defective allosteric feedback inhibition of this enzyme by CMP-NANA, resulting in overproduction of cytosolic N-acetylneuraminic acid, followed by massive urinary excretion. Several mutations of this key enzyme in Middle Eastern Jewish patients and in non Jewish patients worldwide causes hereditary

inclusion body myopathy (HIBM), which is a unique group of neuromuscular disorders characterized by adult-onset, slowly progressive distal and proximal muscle weakness, and the typical muscle pathology of inclusion body myositis (2). Based on the X-ray structure of the prokaryotic UDP-GlcNAc 2-epimerase the homologue amino acid residues Lys24, Asp112, Glu134, Asp143 und His220 of the GNE/MNK should form the main part of the putative substrate binding pocket of the epimerase domain (3). Therefore the mutants K24A, D112N, E134Q, D143N and H220N were generated by site-directed mutagenesis. High level overexpression of GNE/MNK was established by using the baculovirus/Sf9 system. Furthermore GNE/MNK enzyme carrying the HIBM causative "Middle Eastern Jewish mutation" M712T (2) was also generated . The catalytic activity and the oligomeric structure of all these mutants were characterized. (1) Hinderlich et al., J. Biol. Chem. 272 (1997) 24313-24318, (2) Eisenberg et al., Nat. Genet., 29 (2001) 83-87, (3) Campbell et al., Biochemistry, 39 (2000) 14993-15001.

(177) **Structural Analysis of the Sialyltransferase Cst-II from Campylobacter Jejuni in the Absence and Presence of A Sialic Acid Substrate Analogue**

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abstract unavailable

(178) **Evaluation of the Specificity Determinants for Lunatic Fringe**

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The Notch protein is a transmembrane receptor that is involved in a wide range of cell fate decisions in metazoans. Mutation of the Notch protein and components of its signaling pathway have been implicated in several human diseases. The extracellular domain of Notch contains 36 tandem Epidermal Growth Factor-like (EGF) repeats, some of which are involved in Notch's interaction with its ligands. 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 modified by the action of Fringe, a fucose-specific β1,3-N-acetylglucosaminyltransferase. Three mammalian Fringes have been identified; Manic, Lunatic, and Radical Fringe. Not all EGF repeats modified by *O*-fucose serve as a substrates for Fringe; Fringe shows specificity for certain EGF repeats. As an example of this, the first EGF repeat of both human clotting factors VII and IX are both known to be modified by *O*-fucose. However, *in vivo*, only the *O*-fucose on factor IX is modified by Fringe even though both proteins are produced in the same cell. Thus, Fringe selectively modifies *O*-fucose on 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 40 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*. Our initial results using this approach will be presented. This work was supported by NIH grant GM61126.

(179) **Molecular Cloning of An Insect Lysosomal α-Mannosidase cDNA and Characterization of the Gene Product**

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The α-mannosidases remove α-linked mannose residues from the terminal, nonreducing positions of oligosaccharides during either biosynthesis or degradation of N-glycoproteins. While several insect genes encoding α-mannosidases involved in glycoprotein biosynthesis have been isolated and characterized, there are no reports to date of insect genes encoding catabolic α-mannosidases. The best-characterized catabolic α-mannosidases are lysosomal enzymes with acidic pH optima that are inhibited by swainsonine. Thus, the purpose of this study was to isolate a lepidopteran insect lysosomal α-mannosidase cDNA and confirm its identity by characterizing the gene product. A degenerate PCR approach yielded a putative class II α-mannosidase cDNA and bioinformatic analysis indicated that it probably encoded a lysosomal α-mannosidase. This tentative conclusion was supported by expression, purification, and biochemical analysis of a glutathione-S-transferase-tagged form of the cDNA product, as the product cleaved p-nitrophenyl-α-D-mannopyranoside with an acidic pH optimum and this activity was inhibited by swainsonine. Interestingly, the insect lysosomal α-mannosidase was strongly activated by zinc. Confocal microscopy indicated that a green fluorescent protein-tagged form of the cDNA product co-localized with a lysosomal dye. Thus, this is the first report of an insect lysosomal α-mannosidase gene supported by a detailed characterization of the gene product.

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(180) **Identification of A Novel Enzyme Responsible for *O*-fucosylation of Thrombospondin Type 1 Repeats.**

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Epidermal Growth Factor-like (EGF) repeats and Thrombospondin Type 1 repeats (TSRs) are two types of protein motifs known to be *O*-fucosylated. The enzyme responsible for the addition of *O*-fucose to EGF repeats, protein *O*-fucosyltransferase 1 (*O*-FucT-1), has been identified and has been shown to be essential in Notch signaling (Okajima and Irvine, 2002 *Cell* **111:**893; Shi and Stanley, 2003 *Proc. Natl. Acad. Sci. USA* **100:**5234, Sasamura *et al*., 2003 *Development* IN PRESS). TSRs are protein motifs found in many extracellular matrix proteins and are involved in protein-protein interactions. They are approximately 60 amino acids in length and can be identified by a number of conserved residues including six conserved cysteines. We investigated whether *O*-FucT-1, or a different enzyme, adds *O*-fucose to TSRs. We developed an *in vitro* assay to detect *O*-fucosylation of TSRs using bacterially-expressed TSR3 from human thrombospondin-1 and GDP-[^{3}H]fucose. Interestingly, purified *O*-FucT-1 does not *O*-fucosylate TSR3, and extracts of mouse embryonic stem cells lacking *O*-FucT-1 possess *O*-fucosyltransferase activity for TSR3 but not for EGF repeats. These results clearly suggested the existence of a TSR-specific *O*-fucosyltransferase distinct from *O*-FucT-1. Database analysis has identified a putative *O*-fucosyltransferase (*O*-FucT-2) closely related to *O*-FucT-1 in both *Drosophila* and humans (Roos *et al.* 2002 *J. Biol. Chem.* **277:**3168). To elucidate if *O*-FucT-2 is capable of adding *O*-fucose to TSR3, we expressed a soluble form of *Drosophila O*-FucT-2 in *Drosophila* S2 cells and purified it from the medium. The purified enzyme was used in *in vitro* assays with either an EGF repeat or TSR3 as substrate. *Drosophila O*-FucT-2 displayed *O*-fucosyltransferase activity only with TSR3. Therefore we have identified *O*-FucT-2 as a new protein *O*-fucosyltransferase that transfers fucose to TSRs and not to EGF repeats. This work was supported by NIH grants GM61126 to RSH and CA95022 to PS.

(181) **Effects of ?Asn56 Oligosaccharide Structure on Equine Luteinizing Hormone and Follicle-Stimulating Hormone Hybrid Conformation and Receptor-Binding Activity**

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The glycoprotein hormones, luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and chorionic gonadotropin (CG), are cystine knot growth factor superfamily members

composed of a common ? subunit non-covalently associated with a hormone-specific ? subunit. The cystine knot motifs in both subunits create two hairpin loops, designated L1 and L3, on one side of the knot, with the intervening long loop, L2, on the opposite side. A fourth, ? subunit-specific loop embraces the α subunit L2 loop including a critical, N-linked oligosaccharide. Subunit dissociation and reassociation involve threading the ? subunit L2 loop and oligosaccharide through the ? subunit cystine noose. In the human (h) hCG and hFSH 3D structures, the ?L2 loop secondary structure consisted of a ? strand followed by the only consensus ? helix, followed by a second ? strand. All hCG? L2 loop secondary structure was lost following subunit dissociation, while L1 and L3 secondary structure was retained. Small differences in the amount of helical structure reported for hCG and hFSH are potentially significant because the helical region contains a GalNAc transferase recognition site that is buried in hFSH, but accessible in hCG, hLH, and hTSH. Because circular dichroism is exquisitely sensitive to changes in ? helix, we employed it to study changes in horse gonadotropin conformation resulting from changes in oligosaccharide structure. Oligosaccharides differentially affected the receptor-binding activity of the equine (e) gonadotropins, eLH and eFSH. As ?Asn56 oligosaccharide size increased from 1622 to 2327 mass units, LH receptor-binding affinity of LH declined significantly, while the decrease in FSH receptor-binding affinity in FSH was not significant. In the present study, we characterized hormone-specific glycosylation of ?L2 oligosaccharides in eLH?, eFSH?, and eCG? preparations. MALDI mass spectrometry revealed 28-57 structures, including high mannose, hybrid, bi- and triantennary oligosaccharides. The same intact subunit preparations and their ? L2 loop-deglycosylated derivatives were combined with either eLH? or eFSH? to create a dozen hybrid hormone preparations and the CD spectrum for each was determined. We predicted that hybrid hormone preparations obtained by combining intact eLH?, eFSH?, and eCG? preparations with eLH? might exhibit differences in conformation that would disappear when the ?L2 oligosaccharide attached to ?Asn56 was removed by selective peptide-N-glycanase digestion (N56dg-?). CD data supported the first prediction, however, elimination of ?L2 oligosaccharide altered the conformation of each eLH? hybrid, actually increasing the conformational differences between them. The intact ? subunit:eFSH? hybrids had virtually identical CD spectra, as expected. However, the N56dg-?:eFSH? hybrid spectra differed from each other. Thus, differences in ?L2 oligosaccharide structure altered the conformation of eLH, while having no detectable effect on eFSH conformation. Elimination of this oligosaccharide altered conformation of all hybrids suggesting that ?Asn82 (located in ?L3) oligosaccharide influenced gonadotropin conformation, however, its effects were masked by the presence of ?L2 oligosaccharide. Increased helical content accompanied by reduced ? strand content, when associated with reduced receptor binding activity, suggested the beginnings of subunit dissociation. Supported by NIH Grant Number P20 RR16475 from the BRIN Program of the National Center for Research Resources.

(182) Structural Studies of Euphorbin, A Lectin That Selectively Binds to Activated Murine Macrophages.

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We previously reported that euphorbin, a D-galactose binding lectin isolated from the latex of *Euphorbia milii* var.*milii*, induces neutrophil migration and homotypic neutrophil aggregation (Dias-Baruffi et al, 2000, Inflam. Res. 12:732-6,). We now describe additional biological and structural features of euphorbin. Euphorbin was isolated from the plant latex by affinity chromatography on a column of immobilized melibiose. The homogeneity of euphorbin preparation was analyzed by silver or coomassie blue stained SDS-PAGE. The specificity for sugars was evaluated by inhibiting the hemmaglutination determined by the lectin. Among several saccharides the most potent inhibitors were GalNAc and melibiose. Euphorbin was assayed in terms of its ability to bind to murine neutrophils and macrophages. Binding only occurred when the cells were harvested from mice peritoneal cavity previously elicited by thioglycollate injection. This binding to pre-activated leukocytes was inhibited in the presence of specific sugar, denoting the involvement of the lectin CRD. Euphorbin has an apparent molecular weight of 60 kDa. Following heat denaturation, monomers of 29 and 31 kDa were detected on SDS-PAGE stained with comassie blue or PAS. These data suggested that euphorbin is a heterodimeric glycoprotein. Electrospray mass spectrometry analysis of intact euphorbin showed an envelope of protonated ions that after MaxEnt1 de-convolution was characterized as provided by three components with 29293; 29365 and 31906 Da. In order to characterize euphorbin, we determined its amino acid composition, N-terminal sequencing by Edman degradation [AFLHSTXIGPNGY] and peptide mass fingerprint (PMF) of the lectin monomers, separated by SDS-PAGE and submitted to in situ trypsin digestion. The PMF of 29 and 31 kDa bands confirmed that the euphorbin is constituted by two non-identical monomers, associated by noncovalent interactions since no cysteine was detected on its amino acid composition. The tryptic peptides derived from individual euphorbin chains were analysed by direct infusion in a nanospray source. Following separation of the digested by reverse phase HPLC, peptides were also analyzed by MS, in order to increase sequencing coverage. The peptide sequencing was deduced from series of *y* and *b* ions generated by collision-induced dissociation (CID). In addition, the peptides were analyzed by MS-parent ion scanning for immonium ions, I/L;W;Y;H and F. Aiming to detect post-translational modification, they were also analysed by MS-parent ion scanning for oxonium ions, m/z 163; 204 and 366, two peptides at m/z 948 and m/z 1100 were considered candidates to be glycosylated and the MS data will be discussed. The acquired N-terminal and internal sequences of 12 tryptic peptides were submitted to EMBL databank using the fast3s algorithm from FASTA software package (Mackey et al. 2002. Mol. Cell. Proteomics 1:139-147). Individual sequences were also searched on NCBI non-redundant through its BlastP algorithm. No significant similarity was found with known structures, suggesting that euphorbin is a new protein that apparently does not fit the seven families classification of plant lectins. Supported by FAPESP, CAPES, CNPq and FAEPA.

(183) Analysis of Oligosaccharides and Glycosylation Sites of β1,6 N-Acetylglucosaminyltransferase V

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N-acetylglucosaminyltransferase V (GnT-V) has 740 amino acids, six putative N-linked sites and catalyzes the addition of a b1,6 GlcNAc to an a1,6 mannose to form tri- and tetra-antennary complexes. Analysis of breast and colon carcinomas have shown increased GnT-V activity and b1,6 branching. Activated oncogenes such as *ras, src* and *her2/neu* appear to regulate and increase GnT-V activity, while GnT-V knockout mice appear to slow the rate of tumor progression. Structure/function studies of GnT-V suggest that glycans are important for activity, while deletion studies have indicated that the minimal catalytic domain contains only the three C-terminal glycosylation sites, ASN 334, 433 and 447. These data indicate it is these three glycosylation sites that are of importance for activity. We used mass spectrometry techniques combined with exoglycosidase digestions of recombinant soluble human GnT-V expressed in CHO cells and purified from concentrated minimal media to identify the N-glycan structures and their sites of expression. N-glycans make up 10-15% of the mass of GnT-V, and analysis of these permethylated glycans using MALDI-TOF MS indicated a range of complex glycans from biantennary to tetraantennary species. The identification of the glycosylated sites was performed by enriching for trypsin-digested glycopeptides, followed by analysis of each fraction with Q-TOF MS. Potential tryptic glycopeptides were identified by comparisons of theoretical masses of peptides with various glycan masses to the masses of the glycopeptides determined experimentally. Of the six putative glycosylations sites, peptides containing sites ASN 334, 433 and 447 were all three identified as being glycosylated. ASN 334 appears to be glycosylated with only a biantennary structure with one or two terminating sialic acids. Sites ASN 433 and 447 both contain structures that range from biantennary with two sialic acids to tetraantennary terminating with four sialic acids. The predominant glycan species found on both of these sites is a triantennary with three sialic acids. ASN 334, 433 and 447 are all located in the region that encodes for catalytic activity.

(184) Structure of the Yeast α1,2-Mannosyltransferase Kre2p/Mnt1 Suggests a Novel Tyrosine-Dependent Catalytic Mechanism

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Kre2p/Mnt1p is a Golgi α1,2-mannosyltransferase involved in the biosynthesis of *S. cerevisiae* cell wall glycoproteins. We present the first structure of a

mannosyltransferase, a member of the GT-15 retaining glycosyltransferase family. The crystal structures of the catalytic domain of Kre2p and its binary and ternary complexes with the donor substrate GDP-mannose/Mn^{2+} and acceptor methyl-α-mannoside have been determined at 2.0Å resolution. The protein has a α/β fold, similar to glycosyltransferases of the GT-A family despite the absence of sequence homology, with a central seven-stranded mixed β-sheet and a three-stranded anti-parallel β-sheet forming a wedge-like sandwich flanked by α-helices. Manganese is bound by a modified DxD motif (EPD) with only the glutamate involved. The GDP moiety of the donor is tightly bound to the active site, but the mannose moiety is not visible in the electron density. The O2 of the acceptor mannoside forms a hydrogen bond with the hydroxyl of Tyr 220 that, in turn, coordinates a β-phosphate oxygen of GDP to which the presumably cleaved mannose moiety was bound. The donor mannose was modeled using binary complex structures of other GT-A enzymes. The C1 of the modeled donor mannose is within hydrogen-bond distance of the Tyr 220 hydroxyl and its β-linked hydrogen points roughly towards the tyrosine. Combined with modeling, the structures suggest that the hydroxyl of Tyr 220 is the potential nucleophile which may form the covalent β-linked intermediate with the donor mannose before the nucleophilic attack by the acceptor, thereby supporting a double-displacement mechanism of retention for these mannosyltransferases. The Y220F mutation yielded an enzyme with a very low residual activity thus confirming the important role of Tyr 220 in catalysis. Supported by NIH grant GM31265.

(185) **Decreased Monoclonal IgG_1 Galactosylation at Reduced Dissolved Oxygen Concentration Is Not A Result of Lowered Galactosyltransferase Activity *In Vitro***

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Monoclonal antibodies (mAbs) are exceptionally important as *in vitro* and *in vivo* diagnostic reagents, therapeutic pharmaceuticals, and ligands for affinity purification techniques. The majority of mAbs are of the immunoglobulin G (IgG) class and are produced in hybridoma or recombinant cell cultures. Many different aspects of cell culture have been shown to influence glycosylation of mAbs and recombinant glycoproteins, and, thus, their physicochemical, structural, and functional properties.

We have previously reported that the N-linked glycosylation of a monoclonal IgG_1 produced by a hybridoma grown in serum-free continuous culture is dependent upon both the steady-state dissolved oxygen (DO) concentration and the configuration of the bioreactor. More specifically, the level of galactosylation was reduced above and below 100% DO, producing an optimum DO concentration for maximum galactosylation – and dropped considerably between 50 and 10% DO. This effect was more pronounced in one bioreactor than the other, probably due to differences in bioreactor configuration and DO monitoring and supply strategies. The level of galactosylation of the Fc N-glycans at Asn-297 of each of the two heavy chains has particular structural and functional relevance for IgG.

To further investigate the decrease in galactosylation between 50 and 10% DO setpoints, and to determine whether the decrease occured at a specific %DO threshold or as a gradient, we examined N-glycans of the same mAb produced in 1, 2, 5, 10, 25, and 50% DO. A gradual but steep decline in galactosylation was confirmed, which became even more pronounced below 10% DO. We also measured β1,4-galactosyltransferase (β1,4Gal-T) activities of the respective hybridomal cell homogenate supernatants by Gal transfer from UDP-^{3}H-Gal donor to GlcNAc acceptor. The β1,4Gal-T specific activities were remarkably similar in all six cultures and not correlated to the DO concentrations. The overall mean specific activity compared extremely well with activities determined for other B-lymphocyte cell lines using both GlcNAc and macromolecular acceptors.

In rheumatoid arthritis, reductions in galactosylated IgG have been attributed to decreases in β1,4Gal-T activities in peripheral B cells, but not to levels of β1,4Gal-T mRNA or protein. Unlike rheumatoid arthritis, this work has disqualified alterations in *in vitro* β1,4Gal-T activity as the cause of the observed DO effect on galactosylation. This implies that the activity of β1,4Gal-T may be regulated post-translationally by reversible serine phosphorylation, or altered glycosylation, disulfide bond formation, or protein folding. Other physicochemical or biochemical for the observed effect must be explored. We have suggested that the DO effect may reflect a perturbation in the redox state of the ER and/or Golgi, affecting both the rate and timing of formation and shuffling of disulfide bonds – thereby altering access to the Asn-297 N-glycans by the nascently folding protein.

(186) **A Human Homologue of Yeast Smp3p Adds A Fourth Mannose to Yeast and Mammalian Glycosylphosphatidylinositol Precursors *In Vivo***

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Glycosylphosphatidylinositols (GPIs) are essential glycolipids synthesized by all eukaryotes. GPIs become attached to the carboxy-termini of various secretory proteins and anchor them to the exterior face of the plasma membrane. Many of the steps and enzymes in *Saccharomyces cerevisiae* and mammalian GPI precursor assembly are highly conserved. In yeast, late-stage intermediates in GPI precursor assembly, including the presumed GPI transamidase substrate, have four mannoses, whereas the potential GPI transamidase substrates that can be radiolabeled in cultured mammalian cells contain only three. Thus, the fourth mannose (Man-4) represents a notable structural difference between mammalian and yeast GPIs and its addition may be a suitable target for anti-fungal drug development.

In *S. cerevisiae*, Man-4 is added to trimannosyl-GPIs (Man_3-GPIs) by the essential Smp3 α-mannosyltransferase during precursor synthesis (1). Its addition is required for subsequent attachment of EthN-P to Man-3 through which the GPI is ultimately attached to protein (1). Thus, it is likely that all yeast GPIs contain Man-4 upon addition to protein. Conversely, mammalian precursors and most protein-bound GPIs contain three mannoses, suggesting that formation of Man_4-GPIs is not a prerequisite for GPI attachment to protein in mammals. However, Man_4-GPIs are made in mammalian cells. Man-4 is present on the GPIs of the rat brain Thy-1 protein (2) and human renal dipeptidase (3). Additionally, trace amounts of GPI intermediates with chromatographic mobilities consistent with the presence of Man-4 are observed in some cell lines (4). In this study, we sought to identify the protein responsible for Man-4 addition to human GPIs.

A candidate human Man-4 α-mannosyltransferase (hSmp3p) was identified by database searching with the ScSmp3p sequence as a probe (1). A *hSMP3* cDNA was cloned and expressed in *S. cerevisiae* to test its ability to relieve the growth and biochemical defects of *smp3* mutants. Expression of hSmp3p restored viability to yeast cells harboring a lethal *smp3* null mutation and corrected a cell wall synthesis defect in a conditional *smp3* mutant. Additionally, expression of *hSMP3* in a *smp3/gpi13* double mutant led to conversion of Man_3-GPIs to Man_4-GPIs *in vivo*. Immunolocalization experiments showed that epitope-tagged Smp3p resides in the endoplasmic reticulum (ER) in human cells. Northern blot analysis of mRNA from 58 human tissues showed that *hSMP3* is weakly expressed in most tissues, with the highest levels in brain and colon. Interestingly, *hSMP3* expression was undetectable in human carcinoma cells (HeLa) and other cultured cell lines. Overexpression of *hSMP3* in human HeLa cells resulted in the formation of two putative Man_4-GPIs. Structural characterization of these lipids is underway. These data are consistent with the notion that *hSMP3* encodes a GPI mannosyltransferease able to add Man-4 to yeast and likely to human GPIs.

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(187) **Initiation of Mucin-Type O-Glycosylation in Lower Eukaryotes (O-α-GlcNAc-type) and Higher Eukaryotes (O-α-GalNAc-type) Is Homologous**

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Mucin-type O-glycosylation, the kind that is initiated with GalNAc in α-linkage to the hydroxyl of Thr- or Ser-residues, is a major modification of animal glycoproteins. These modifications are often clustered on amino acid

repeats that are rich in Thr, Ser, Gly, Pro and Val, as occurs in mucin proteins. It now appears that some groups of lower eukaryotes also carry out mucin-type O-glycosylation. It has been known for some time that the protozoan Trypanosoma cruzi and the mycetezoan Dictyostelium discoideum form GlcNAcα1-Thr linkages in mucin-like peptide repeats. But a mechanistic relationship to animal mucin-type O-glycosylation was not apparent. The gene for the soluble polypeptide N-acetyl-α-glucosaminyltransferase (pp αGlcNAcT) that forms a similar GlcNAcα1-HyPro linkage on the cytoplasmic/nuclear glycoprotein Skp1 has been cloned. tBLASTn searches seeded with the Dd-ppαGnT1 catalytic domain yield three classes of related sequences in other organisms: 1) other predicted soluble cytoplasmic pp αGlcNAcTs in cyanobacteria, proteobacteria, and a diatom, 2) putative membrane-bound Golgi pp αGlcNAcTs in lower eukaryotes including trypanosomatids, a diatom and Dictyostelium, and 3) the well-known Golgi pp αGalNAcTs that initiate mucin-type O-glycosylation in animals and apicomplexans (Toxoplasma). To test the function of a class 2 sequence, an example from Dictyostelium was expressed recombinantly and its gene was disrupted, leading to the conclusion that it is a bona fide pp αGlcNAcT for cell surface proteins in this organism and important for several developmental processes. Interestingly, this enzyme (Dd-ppαGnT2) is inhibited by two cell-permeant uridine derivatives from a screen by Bertozzi et al. for uncharged inhibitors of the class 3 enzyme murine pp αGalNAc-T1, suggesting that the class 2 and class 3 enzymes are homologous. The pp αGlcNAcT activity of T. cruzi microsomes is also affected by these inhibitors, and therefore a related class 2 sequence in the genome of this organism is a prime candidate for encoding this enzyme. A phylogenetic analysis traces the origins of the three classes of sequences back to predicted gene duplications in prokaryotes. These findings suggest an ancient history for the evolution of mucin-type O-glycosylation in prokaryotes and lower eukaryotes, like that recently found for N-glycosylation, and provide new approaches for the functional analysis of O-glycosylation in parasites and pathogens. (H. Hang & C. Bertozzi are thanked for providing inhibitors; S. & H. Alexander are thanked for the cis4c-mutant; supported by NIH & NSF)

(188) **Measurement of Cerebroside Sulfate Activator Activity by Multiple Reaction Monitoring**

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Introduction

Humans deficient in the cerebroside-sulfate activator protein (CSAct or Saposin B) are unable to catabolize sulfatide (cerebroside sulfate, CS) leading to its accumulation and neurodegenerative disease (Li, S. C. et al, *J Biol Chem.* **1985**, 260, 1867-1871). Clinically, this usually manifests as a form of Metachromatic Leukodystrophy (MLD). CSAct is a small water-soluble glycoprotein that apparently functions in the lysosome to solubilize CS and other lipids, enabling their interaction with soluble lysosomal hydrolases. CSAct activity can be measured *in vitro* by assay of its ability to activate sulfatide-sulfate hydrolysis by arylsulfatase A (ASA). Existing protocols require radio-labeled sulfatide, prepared *in vivo* from $^{35}SO_4^{2-}$ administration to the brains of experimental animals followed by extraction and purification. Here we present a novel approach to the analysis of the ASA-Saposin B system.

Methods:

Electrospray ionization (ESI) mass spectrometry coupled to tandem mass spectrometry (MS/MS) using multiple reaction monitoring (MRM) was used to follow the appearance of the cerebroside product, rather than the inorganic sulfate product monitored previously. Detection of the neutral glycosphingolipid cerebroside was achieved via enhancement of ionization efficiency observed in the presence of Li^+ (Hsu and Turk, **2001**, *J. Am. Soc. Mass. Spectrom.* 12, 61-79). Assay samples were extracted into chloroform as for the existing assay (Whitelegge et al, **2000**, Protein Science 9, 1618-1630), dried and diluted in methanol/chloroform (4:1; v/v) containing 100 mM LiCl. Samples were analyzed by positive ion ESI with a triple quadrupole mass spectrometer (API III, Applied Biosystems, Norris et al, **2001**, *Biochemistry* 40, 3774-3779).

Preliminary Results:

ASA activity was tested for dependence on recombinant saposins A, B, C, D and native saposin B from pig kidney. ASA activity was first assessed by monitoring multiple products simultaneously from the metabolism of bovine CS in the presence of Saposin B. Up to 0.6 M NaCl and 5mg/ml detergent was tolerated by the ESI-MS/MS-MRM based assay. In an activity vs. abundance profile of the various CS isoforms using ASA and Saposin B, preliminary findings indicate that there are subtle differences in the metabolism of each isoform with the longer chain fatty acids being metabolized slightly more rapidly. In order to investigate whether other saposins have any activating properties to them with respect to ASA, recombinant Saposins A, B, C, and D, were run under identical conditions. The results indicate that only Saposin B is an activator of ASA, and thus the previously reported activity for SAP C and D may be due to Sap B contamination (Hiraiwa, M. et al. *Arch Biochem Biophys* **1993**, 304, 110-116). The existing assay measures inorganic sulfate formation which represents the bulk rate of CS hydrolysis. Here we demonstrate a method that can not only monitor the metabolism of CS, but can measure the rate of formation of individual cerebroside species. Extrapolation of this method to assays other saposin systems should be possible.

(189) **Novel Glycan Structures on the Endogenous Glycoconjugates of Higher Plants.**

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Plants are emerging as a suitable host system for the production of therapeutically important mammalian proteins. However, one of the shortcomings with the use of plants is insufficient knowledge regarding post-translational modifications (PTMs) that are crucial for the structure and functions of many proteins. Whether or not plants perform mammalian-like glycosylation is emerging as an important question especially with regards to the serum half-life and the functionality of therapeutically important glycoproteins. In studying glycan structures of endogenous glycoproteins of Arabidopsis thaliana, we have discovered mammalian-like glycan motifs such as GalNAc-O- Ser/Thr (Tn antigen), Gal-b1,3-GalNAc-O-Ser/Thr (T antigen) and also previously unreported sialylated glycoconjugates. The presence of sialic acids on A. thaliana glycoconjugates was confirmed using Immunofluorescence imaging, lectin blotting, RP-HPLC and mass Spectrometry. These data suggest that the complex glycosylation (i.e., sialylation) pathway does exist in plants. However, further research is required to understand and manipulate these pathways in plants to produce glycoconjugates with desired glycan motifs.

(190) **O-glycans from Polymeric IgA1 from Serum of Patients with Type 2 Diabetes Mellitus Are Hypersialylated**

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Serum IgA1 is frequently elevated in Type 2 diabetes mellitus patients (DM). Previous studies have shown that O-glycans from IgA1 are hypersialylated and probably implicated in the serum elevation of this protein. Macromolecular IgA1 seem to be the main hypersialylated IgA1 fraction, however other IgA1 fractions may be implicated in this process. The aim of this work was to search for differences in sialylation of O-glycans from monomeric, polymeric and macromolecular IgA1 serum fractions from DM patients. IgA1 fractions were obtained from sera of 9 DM patients and 9 healthy matched controls by a four-step chromatographic procedure, including highly acetylated-Sepharose, anti-IgA-agarose, jacalin-agarose and Bio-Gel A. Analysis of O-linked oligosaccharides from IgA1 was realized by enzyme-linked lectin assay (ELLA) using SNA, MMA, PNA, and jacalin lectins. Additionally, O-glycans from native IgA1 were analyzed by fluorophore assisted carbohydrate electrophoresis (FACE). Monomeric, polymeric and macromolecular IgA1 from DM patients were more reactive to MMA. This indicated the probably elevation of alpha 2,3 sialic acid from all IgA1 fractions. According to previous results, polymeric fraction resulted more reactive to SNA indicating the elevation of the alpha 2,6 sialic acid content. Comparable results were demonstrated by using FACE. This results showed that polymeric IgA1 fraction is the main hypersialylated IgA1 fraction from serum DM patients and probably contributes to the high levels of IgA1 in DM patients.

(191) **Underfucosylated Monomeric Serum IgA1 from Type 2 Diabetes Mellitus Patients**

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Serum IgA1 is frequently elevated in patients affected by type 2 diabetes mellitus (DM). The elevation of this protein in the absence of infection or vascular complications suggested the possibility of structural changes in the molecule. Previous studies dealing with DM patients without complications and infection have shown that macromolecular serum IgA1 from DM patients is hypersialylated and probably contributes to the high levels of IgA1 in DM patients. The present study was carried out to search for differences in glycosylation of serum monomeric IgA1 from DM patients. Immunoglobulin A1 (IgA1) was purified from sera of nine DM patients having high IgA1 serum levels, and from healthy matched controls. A three step chromatographic procedure including highly acetylated-Sepharose 6B and anti-IgA-agarose columns were used to isolate IgA. IgA1 was further purified from IgA using a jacalin-agarose column and further separated into macromolecular, polymeric and monomeric fractions by using gel filtration column. IgA1 fractions were screened for carbohydrate changes, using SNA, MMA, PNA, WGA, LcH, ConA, RCAI, SBA, DBA, LTL, UEA, VVA, AAA, and DBA lectins. Monomeric IgA1 from controls was more reactive to LTL and UEA lectins than matched controls. This indicated that monomeric serum IgA1 from DM patients is probably underfucosylated.

(192) **Interaction of Acharan Sulfate with Plasma Proteins**

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Glycosaminoglycans (GAGs), such as heparin and heparan sulfate, are of great biological importance. Protein-GAGs interactions play prominent roles in cell-cell recognition and cell growth. Acharan sulfate (AS), isolated from the giant African snail *Achatina fulica*, is a novel member of glycosaminoglycan families. It showed antitumor activity by inhibition of angiogenesis. We previously reported that acharan sulfate (AS) showed anticoagulation activity *in vitro*, but it was much less than that heparin. We recently investigated the *in vivo* anticoagulant activity of acharan sulfate. The intravenous administration of acharan sulfate prolonged the clotting time (APTT) in mice and rats in a dose-dependent manner. Although the activity was low in rats, it could be maintained over 5h after administration of AS (30 mg/kg). In contrast, the activity of heparin (5 mg/kg) was restored to the normal level after 3 h. In a thrombin-induced lethality model in mice AS (20 mg/kg) protected the lethality by 80 percent, while heparin (20 mg/kg) did not show any protective activity at 3.5 h after administration. We could also detect the plasma concentration of AS even 5 hours after administration to rats. These results show that the longer duration of AS in blood has a possibility to interact with coagulation factors. Based on these results, AS was immobilized to agarose matrix by EDC/diaminodipropylamine coupling method. The immobilized gel packed in the column was exposed to human plasma. The column was eluted with a stepwise salt gradient (0M, 0.3M, 0.4M, 0.7M, and 1M NaCl in 20mM Tris-HCl). Two proteins, ceruloplasmin and prothrombin, were characterized by SDS-PAGE and MALDI-TOF-MS. From this study, we speculate that the interaction of plasma proteins with AS may be important in exhibiting a longer anticoagulant activity in the body.

(193) **A Novel β (1,6) N-Acetylglucosaminyltransferase V (GnT-VB) Transcript Is Expressed in High Levels in Mouse and Human Brain.**

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UDP-N-acetylglucosamine: α(1,6)-D-mannoside β(1,6)-N-acetylglucosaminyltransferase (GnT-V, Mgat5) functions in the biosynthesis of N-linked glycans and is transcriptionally upregulated by oncogene signaling, causing decreased cell-cell and cell-matrix adhesion that result in increased cell motility and invasiveness. Human and mouse cDNAs encoding a distinct enzyme with related substrate specificity, termed GnT-VB, have been expressed and the enzymatic properties of these proteins characterized. The human and mouse enzymes are predicted to have about 53% similarity to the original amino acid sequences of human and mouse GnT-V(A). Transient expression of human and mouse GnT-VB cDNAs in COS7 cells yielded significant increases of activity toward GnT-VA acceptors, including synthetic saccharides and N-linked glycopeptides, with some differences in specificity compared to GnT-VA. Both GnT-VBs required divalent cation for full activity, while GnT-VAs did not. Human and mouse EST databases showed expression of 6 base-pair splice isoforms of GnT-VB; when recombinantly expressed, these + 6 bp isoforms showed reduced activity. CHO Lec4 cells, which do not express GnT-VA activity, lack synthesis of the N-linked β(1,6) branch, and do not bind L-phytohemagglutinin (L-PHA). When transfected with human GnT-VB or GnT-VA, Lec4 cells bound significant amounts of L-PHA, demonstrating that both enzymes synthesized N-linked β(1,6) branched glycans. Analysis of N-glycanase-released oligosaccharides from these transfected cells confirmed synthesis of tetra-antennary N-linked structures by both GnT-VB and GnT-VA. Real-time PCR results revealed that human GnT-VB mRNA was highly expressed in brain and testis, with lesser levels in other tissues, while human GnT-VA showed a more general expression, but with low levels in brain and no expression in skeletal muscle.

(194) **Characterization of IspC, 1-Deoxy-D-xylulose-5-phosphate reductoisomerase from *Mycobacterium tuberculosis***

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Isopentenyl diphosphate (IPP) is an essential precursor in the biosynthesis of all isoprenoids, an ancient and diverse class of natural compounds. Two distinct routes of IPP biosynthesis occur in nature. Until recently, it was widely assumed that the early steps of IPP biosynthesis described for vertebrates (the mevalonate pathway) also apply to prokaryotes. However, a series of studies have shown that some bacteria including mycobacterial species use the mevalonate-independent or 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway. Since the mevalonate-independent pathway does not occur in mammals, the enzymatic steps involved in this pathway have been identified as potential targets for the development of antibacterial compounds against pathogenic bacteria. 1-Deoxy-D-xylulose-5-phosphate reductoisomerase (*IspC*) is the second enzyme of the MEP pathway. *IspC* catalyzes the committed step involving the conversion of 1-deoxy-D-xylulose-5-phosphate (DXP) to MEP, in a reversible reaction. The two-step conversion involves an intramolecular C-C rearrangement of DXP followed by NADPH dependent reduction to MEP. We have cloned, expressed and characterized *Rv2870c*, which encodes *Mycobacterium tuberculosis* IspC. The enzyme was characterized in both the forward and backward directions. K_m values of 47.1 ± 5.9, 29.7 ± 2.7, 174.0 ± 28.1 and 560 ± 78.0 μM, were determined for DXP, NADPH, MEP and NADP, respectively. We have determined that the enzyme is sensitive to fosmidomycin, (IC_{50} 0.310 ± 0.025 μM), which has antibacterial activity against many Gram-positive and Gram-negative bacteria. However, fosmidomycin does not inhibit mycobacterial growth, and hence this is not an ideal lead compound for further drug development.

(195) **Species Specific Inhibitors of Glycosylphosphatidylinositol Anchor Biosynthesis that Target Trypanosoma brucei, the Causative Agent of African Sleeping Sickness**

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Trypanosoma brucei is a blood dwelling protozoan parasite, transmitted by the Tstse fly, which causes human Sleeping Sickness and diseases in Cattle. The bloodstream form of the parasite evades the host immune system through antigenic variation of a glycosylphosphatidylinositiol (GPI) anchored variant surface glycoprotein (VSG) that forms a dense coat on the exterior surface of the parasite (1), making the production of a vaccine unfeasible. However, a conditional-null mutant of the second step of the GPI pathway (GlcNAc-de-N-acetylase) has shown that this enzyme is essential for the bloodsteam form of the parasite (2). We have been able to exploit differences in the specificity of the parasite and host GlcNAc-de-N-acetylase to make a parasite-specific inhibitor (3).

This poster will summarise this work and present the results of current attempts to express the parasite and host GlcNAc-de-N-acetlyase enzymes for

structural analysis. We hope that crystallographic data on these enzymes with and without bound inhibitors will assist us in refining our inhibitor design.
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(3)Smith, T. K.; Crossman, A.; Borrissow, C. N.; Paterson, M. J.; Dix, A.; Brimacombe, J. S.; Ferguson, M. A. J., *EMBO J.* (2001), 20, p3322-3332.

(196) **Recent Advances for the Analysis of Glycopeptides by Mass Spectrometry**

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There is a growing need to analyze glycoconjugates in biological compounds. Cell surface glycoconjugates are involved in many processes, including cell-cell interaction. However, such compounds are often found in complex mixtures and usually in trace amounts. Therefore highly and selective methods are required for their analysis. We reported our initial effort at evaluation of the conditions for an on-line system of reversed-phase HPLC of oligosaccharides with ion-spray MS and tandem MS (MS/MS)1. This analysis method was rapid and simple, as the mass spectrometer was able to give the accurate molecular weight of each oligosaccharide in one chromatography run, even if the HPLC separation was incomplete. Two-dimensional gel electrophoresis (2-DE) is a protein separation technique that combines two different electrophoretic methods, which is gel isoelectric focusing (IEF) in the first dimension (in which proteins are separated according to pI) and SDS-PAGE in the second dimension (separation according to molecular weight). In proteome research by using 2-DE and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI/TOF MS) for protein identification has used widely. This is the most effective method for checking of protein expression regulation totally. We will present the study on direct analysis of glycopeptides, which has been ignored from MASCOT analysis, derived from 2-DE separated and in-gel digested proteins by using mass spectrometry. We will show glycopeptides are useful molecules for both peptide sequence analysis amd oligosaccharide structure analusis. This work was partially supported by the 21 COE program supported by the Ministry of Education, Science, Culture, Sports and Technology Japan and CREST of JST (Japan Science and Technology Corporation). References 1. Sawada-Suzuki, J., Umeda, Y., Kondo, A., and Kato, I. (1992) Anal. Biochem. 207, 203-207.

(197) **Change in Glycosylation of Vitronectin Modulates the Tissue Lytic System and Remodeling During Liver Regeneration and Cirrhosis.**

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Vitronectin (VN) is a multifunctional glycoprotein present in plasma and the extracellular matrix (ECM); it is the only ligand that can stabilize plasminogen activator inhibitor-1 (PAI-1) in ECM. PAI-1, which is one of the most important regulatory factors in matrix degradation, suppresses plasmin generation. The active form of PAI-1 is unstable *in vivo*, but it is stabilized by forming a complex with VN. Previously, we reported that glycosylation of VN changes significantly during liver regeneration[1)] and hepatic inflammation[2)], and influences tissue remodeling by changing the collagen binding of VN. In this study, changes in the interaction of VN with factors in the tissue lytic system were analyzed to elucidate the effects of the change in glycosylation of VN during liver regeneration and cirrhosis.

The PAI-1 binding activity of VN purified from rat plasma was decreased to 1/3 and 2/3 of that of non-operated (NO) rats 24 h after partial hepatectomy or sham-operation, respectively. Upon glycosidase-digestion of VNs, PAI-1 binding activity was enhanced significantly by de-*N*-glycosylation but not by desialylation. These results suggest that the PAI-1 binding activity of VN changes with the glycosylation and that the attenuated PAI-1 binding activity in the early stage of liver regeneration is due to the change in glycosylation of VN.

On the other hand, plasma concentrations of VN in liver cirrhosis (LC) declined to 2/3 that in normal plasma, but the VN that is active in collagen binding increased in cirrhotic plasma whereas most VN is inactive in normal plasma. The active VN was found to have a lower molecular weight than that of inactive VN. After de-*N*-glycosylation, both VNs migrated to the same position on SDS-PAGE suggesting that the active VN is underglycosylated. Because the increase of the active VN in LC plasma may contribute to matrix incorporation of VN, VN in cirrhotic tissue would have enhanced PAI-1- and collagen-binding activities. Therefore, the glycosylation change of VN may account for the positive correlation of collagen-binding VN in plasma and the progression of liver cirrhosis[3)].

1) Uchibori-Iwaki H *et al* (2000) *Glycobiology* **10**:865-874
2) Suzuki R *et al* (2001) *Int. Congr. Ser.* **1223**:103-107
3) Yamada S *et al* (1996) *Clin. Chem. Acta* **252**:95-103

(198) **Comparison of Novel Carbohydrate Binding Activities of Porcine Pancreatic α -Amylase and Recombinant Human Pancreatic α -Amylase**

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Pancreatic α-amylase typically cleaves α-1,4-glucose linkages of starch in the small intestine. Recently we found a novel binding activity of porcine pancreatic α-amylase (PPA) toward *N*-linked oligosaccharides of glycoproteins[1)]. In this work, the carbohydrate-binding activity of human pancreatic α-amylase (HPA) was examined in comparison with PPA to study the biological significance of sugar-specific interaction, which is different from substrate recognition.

Porcine pancreatic α-amylase was shown by interaction analyses using a resonance mirror detector and α-amylase-immobilized Sepharose to bind with glycoproteins possessing *N*-glycans, but not *O*-linked mucin-type glycans. Direct binding of three types of *N*-glycans to the α-amylase was demonstrated by surface plasmon resonance (SPR). Binding with biotin-polymer (BP-) sugar probes revealed that the α-amylase has affinity to α-mannose, α-*N*-acetylneuraminic acid, and *N*-acetyllactosamine, which are components of *N*-glycans. The binding of glycoproteins or carbohydrates enhanced the enzyme activity, indicating that the recognition site for *N*-glycans is different from its catalytic site. The binding activity was unique to PPA and not observed for α-amylase from human saliva, wheat or fungus.

The carbohydrate-binding activity of recombinant HPA (recHPA) was examined. RecHPA was produced by inserting HPA cDNA into yeast, *Saccharomyces cerevisiae* W303-1A, transfected with the YEp51 plasmid. RecHPA was purified from the culture medium by ammonium sulfate fractionation, followed by affinity chromatography (AFC) on an amylose-Sepharose column, and eluted with maltose. The carbohydrate binding activities of HPA and PPA were first compared by affinity chromatography on a Man-Sepharose or a Gal-Sepharose column. PPA bound to both columns and was eluted with Meβ-Gal better than with Meα-Man, indicating that PPA has affinity to β-Gal as well as to α-Man. When recHPA was applied to a Gal-Sepharose column, it bound to the column and was eluted with Meβ-Gal like PPA. The binding studies with BP-sugar probes indicated that recHPA exhibits binding to *N*-acetyllactosamine, α-*N*-acetylneuraminic acid, and α-mannose-6-phosphate. The results indicate that the carbohydrate-binding activity is common to PPA and recHPA and carries out a pancreas-specific function.

The endogenous glycoreceptors for pancreatic α-amylase are being screened using labeled PPA and recHPA as probes, which will reveal the biological function of the novel carbohydrate-binding activity.

1)Matsushita *et al. J. Biol. Chem* (2002) **277** 4680-4686

(199) **Aberrant Expression of α-Gal on Primary Human Endothelium Does Not Confer Susceptibility to NK Cell Cytotoxicity or Increased NK Cell Adhesion**

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Abstract unavailable

(200) **Cleavage of Galectin-3 by Neutrophils; Regulation Mechanism for Oligomerization-Driven Activities**

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Galectin-3 is suggested to play a role as an immunomodulator for various innate immune responses. Most of galectin-3's roles in those immune

responses such as signal transduction in cell, cell-cell interactions and cell adhesion, are dependent on the oligomerization of galectin-3 molecules, which consists of a carbohydrate recognition domain (CRD) linked to a N-terminal, non-lectin, domain that can oligomerize upon carbohydrate recognition by the CRD. Our laboratory has recently proposed a role for galectin-3 as an adhesion molecule in the β_2-integrins independent neutrophil recruitment during streptococcal pneumonia. Considering the importance of galectin-3 functions in a variety of immune responses, such as neutrophil recruitment, regulation of these activities is likely to be required. One way in which oligomerisation driven galectin-3 activities could be regulated is through proteolytic cleavage of galectin-3 between its two domains, the CRD and the N-terminal domain. Galectin-3 cleavage generates a dominant negative form of galectin-3 consisting of the CRD domain but lacking the N-terminal domain and could thus prevent galectin-3's oligomerization driven function. It has been shown that purified bacterial collagenase and metalloprotease 2 and 9 can cleave galectin-3, although whether such cleavage takes places in cells has not been well investigated. In this study, we found that the dominant negative form of galectin-3 is generated in vivo during streptococcal pneumonia. Our in vitro data using cells which are potentially involved in the cleavage of galectin-3 suggest that primed neutrophils are mainly responsible for the cleavage in vivo. Further more, in the case of neutrophil, elastase rather than the metalloproteases is implicated in the cleavage. We thus propose that the formation of the dominant negative form of galectin-3 could represent a regulatory event of immunological activities of galectin-3 such as galectin-3 mediated adhesion.

(201) **P-Selectin Ligand Formation in Core 2 β 1-6 N-Acetyl Glucosaminyltransferase-1^{null} CD8 T cells**

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Core 2 β 1-6 *N*-acetyl glucosaminyltransferase (C2GnT) is a key enzyme required for the formation of core 2 branched O-glycans including ligands for selectin mediated cell-cell adhesion in lymphocyte trafficking and inflammation. Three C2GnT isoenzymes have been identified to date: C2GnT1, C2GnT2 and C2GnT3. C2GnT1 is the most thoroughly studied of the three enzymes; it is both widely expressed and involved in the synthesis of P-selectin ligands (P-selL) in leukocytes. Much less is known about C2GnT2 and C2GnT3. In humans C2GnT2 is expressed in mucous epithelial cells where it participates in mucin production while C2GnT3 expression is restricted to the thymus that may reflect a unique role of this enzyme during T cell development. We are interested in determining the role of C2GnT2 and C2GnT3 in selectin mediated cell-cell adhesion. Analysis of activated CD8 T cells from C2GnT1^{null} mice revealed that P-selL formation occurred in these cells in absence of the C2GnT1 enzyme. Induction of P-selL formation was cell density and cytokine dependent. P-selectin binding could be inhibited with the neutralising anti-PSGL-1 antibody, 2PH-1, confirming PSGL-1 as P-selL. Up-regulation of P-selL correlates with an increase in core 2 activity as measured by a standard core 2 enzyme assay and by cell surface binding of the core 2 sensitive monoclonal antibody, 1B11. CD8 T cells from C2GnT1^{null} mice that showed P-selL expression were capable of rolling under shear flow on immobilised P-selectin.

To assess whether P-selL induction is supported in C2GnT1^{null} CD8 T cells in vivo, we analysed T cells from mice transgenic for the male antigen T cell receptor (HY). CD8 T cells from female HY^{tg} C2GnT1^{wt} control and female HY^{tg} C2GnT1^{null} mice were transferred into male recipient mice. CD8 T cell response and P-selL induction was measured 2-4 days after cell transfer. Both wt and C2GnT1^{null} CD8 T cells showed comparable proliferative response. While wt controls expressed high levels of P-selL, C2GnT1^{null} cells were also positive for P-selL, although at reduced levels compared to wt cells. Our findings thus implicate C2GnT2 and/or C2GnT3 as possible players in core 2 branch formation on PSGL-1. While these enzymes have already been implicated in the human system to be involved in core 2 branch formation of the leukocyte mucin CD43, our data are the first to associate these alternate core 2 enzymes with P-selL formation.

(202) **Causal Involvement of Macrophage Galactose-Type C-Type Lectin 1 (MGL1) in the Granulation Tissue Formation**

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Macrophages are known to play major roles at the first and the final stages of the cellular immune response. It is well known that macrophages have antigen presenting finctions. Also, it is believed that these cells play active roles in the inflammatory tissue formation and tissue remodeling. However, regulatory molecules at these late stage of cellular immune response were not thoroughly investigated. In the present report, the role of a macrophage galactose-type calcium-type lectin 1 (MGL1) in antigen-specific chronic inflammatory tissue formation was investigated. Repeated stimulation with a specific immunogen, azobenzene arsonate-conjugated acetylated BSA in dorsal air pouches generated on the back of mice resulted in a persistent presence of extensive granulation tissue, only in wild-type mice, but not in MGL1-deficient mice. Serum IgG antibody levels specific for this antigen was almost identifical between wild-type and MGL1-deficient mice. Furthermore, antigen-specific proliferation of splenocytes showed similar dose response curves for the immunogen between wild-type and MGL1-deficient mice. These results indicate that MGL1 is causally involved in the tissue remodeling phase, but not in the sensitization phase of the antigen specific cellular immune response. This is the first report to show that a cell surface lectin on macrophages plays a significant role in the regulation of antigen specific inflammatory tissue generation.

(203) **Controlled Conjugation of Synthetic Vibrio Cholerae O:1 Antigens Yields Series of Immunogens with Predetermined Carbohydrate-Protein Ratios**

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Cholera is a serious enteric disease for which a satisfactory vaccine is not available. The main causes of cholera are two strains of Vibrio cholerae O:1, Inaba and Ogawa. The protective antigens are the O-polysaccharides (O-PS) of Vibrio cholerae, which consist of (1-2)-linked α-D-perosamine whose amino groups are acylated with 3-deoxy-L-glycero-tetronic acid. The terminal perosamine residue in the Ogawa O-PS carries a methyl group at O-2, which is not present in the Inaba O-PS. In continuation of our work towards a conjugate vaccine for cholera, we have prepared di- through the pentasaccharide that mimic the upstream terminus of the O-specific polysaccharide of Vibrio cholerae O:1, serotype Ogawa. The oligosaccharides were synthesized in the form of 5-methoxycarbonylpentyl glycosides and linked to BSA using squaric acid diester chemistry. The conjugation reactions were monitored by Surface Enhanced Laser Desorption-Ionization-Time-of Flight Mass Spectrometry (SELDI-TOF MS) in combination with the ProteinChip® System. This technique has brought the process of making neoglycoconjugates to a new level of sophistication, as it can provides near real-time information about the progression of the conjugation, in a way similar to what thin-layer chromatography does in organic synthesis of small molecules. Thus, conjugation of synthetic oligosaccharides can be conducted in controlled way and the reaction can be terminated when the desired molar hapten-BSA ratio had been reached. We have now developed a protocol that makes it possible to prepare, from one hapten in a one-pot reaction, a series of neoglycoconjugates having different, predetermined carbohydrate-carrier ratios. In addition, compared to previous monitoring of conjugation of carbohydrates to proteins through SELDI-TOF MS, the accuracy of molecular mass reading could be increased by using the carrier protein as the internal standard. The use of internal standard provides more reliable results and makes the fine calibration of the instrument virtually unnecessary.

(204) **Sialoadhesin in the Subcapsular Sinus of Murine Lymph Nodes Acts as a Ligand for Macrophage Galactose-type C-type Lectin 1 (MGL1)**

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BACKGROUND

Migration of cells in the immune system is critical in the regulation of the immune response. During the sensitization phase of contact hypersensitivity, epidermal Langerhans cells and macrophages in dermis migrate from the skin to the regional lymph nodes (LNs) after percutaneous invasion by an antigen. However, contribution of adhesion molecules to the skin-to-LN migration of putative antigen presenting cells is poorly understood.

MGLs (macrophage galactose-type C-type lectins), MGL1 and MGL2 at least in mice, are family members of C-type lectins and expressed on macrophages in dermis. These MGL1/2+ cells in the dermis were previously shown to migrate into and accumulate within regional LNs after sensitization with FITC dissolved in an acetone/dibutylphthalate (1/1) mixture. The immunohistochemical distribution of MGL1/2+ cells in regional LNs after sensitization was highly restricted to the subcapsular and medullary sinus, interfollicular regions, and the boundary between T- and B-cell areas. The distribution of MGL1/2+ cells was overlapped with that of the areas bound by recombinant MGL1 (rMGL1) in frozen sections of LNs. These results suggested that the unique distribution of MGL1/2+ cells in LNs was determined by the interaction between MGL1 and its ligands. As an initial step to prove this hypothesis, we demonstrated that sialoadhesin (Sn) acted as an endogenous ligand for MGL1 in LNs by a combination of biochemical experiments including affinity chromatography, MS-fingerprinting, and Western blotting. In the present report, we focused on subcapsular sinus and performed immunohistochemical examinations to determine whether distribution of Sn coincided with that of MGL1 ligands.

SPECIFIC AIM

To test the distribution of Sn, MGL1, and rMGL1-binding sites in subcapsular sinus of LNs and to correlate time-dependent localization of MGL1+ cells after epicutaneous administration of antigens.

METHODS

Frozen sections of brachial LNs were treated with biotinylated rMGL1 or mAb LOM-8.7 (anti-MGL1 mAb), and subsequently with Alexa 568-conjugated streptavidin or FITC-conjugated mouse anti-rat κ/λ light chains respectively. In the case of LNs sensitized with FITC, Cy5-conjugated mouse anti-rat IgG was used as the second antibody for mAb LOM-8.7. The same sections were stained with anti-Sn mAb (3D6 or biotinylated SER-4), followed by staining with FITC-conjugated mouse anti-rat κ and λ or Alexa 568-conjugated streptavidin. The sections were observed under a confocal microscope.

RESULTS AND DISCUSSION

A small number of MGL1+ cells revealed by the binding of mAb LOM-8.7 co-localized with Sn+ cells identified by mAb 3D6 or SER-4 binding before the administration of FITC solution, though the incidence was limited. The number of MGL1+ cells around subcapsular sinus increased after sensitization with FITC. At 12 or 24 hours after the sensitization, some of the MGL1+ cells co-localized with Sn+ cells at the inner side of the subcapsular sinus. MGL1+ cells outside the subcapsular sinus did not co-localize with Sn+ cells. A part of the MGL1+ cells appeared to dissociate from the layer of Sn+ cells and to migrate inside at 24 hours. These observations indicated that Sn acts as a ligand for MGL1 within subcapsular sinus. The interaction seems to restrict the migration of MGL1+ cells in these areas.

(205) **Mechanisms of Siglec-8-Induced Human Eosinophil Apoptosis and Regulation by Interleukin-5 (IL-5)**

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Recently, we demonstrated a novel apoptosis-inducing function of Siglec-8 on eosinophils (Blood 101:5014, 2003), and priming with survival-promoting cytokines (i.e., IL-5, GM-CSF) potentiated this effect, unexpectedly. Intracellular pathways involved in Siglec-8-mediated apoptosis are unknown. Decisive events during apoptosis include loss of mitochondrial membrane potential, cytochrome (cyt)-c release and caspase activation. Therefore, we explored mechanisms by which Siglec-8-crosslinking induces purified human eosinophil apoptosis in the presence or absence of IL-5. Caspase-3 activity was measured using the EnzChek assay system. In parallel experiments, caspase-3, -8 and -9 cleavage was investigated by western blotting using specific antibodies recognizing both pro- and cleaved (i.e., active) caspase forms. Viability was investigated using annexin-V staining. mitochondrial membrane potential was assessed by tetramethylrhodamine ethyl ester (TMRE) and cyt-c staining. Our results demonstrated that after 6-18 hours of eosinophil culture, caspase-3 activation was markedly augmented by Siglec-8 crosslinking compared to control cells (1030±300 versus 467±73 pmoles at 18 hours, $p < 0.05$, n=4). Preincubation of eosinophil protein extracts with the caspase-3 inhibitor Ac-DEVD-CHO abrogated caspase-3 activation. Caspase-3 activation correlated with apoptosis (as assessed by annexin-V staining) as well as cleavage of caspases-8 and -9. IL-5 did not prevent Siglec-8-dependent caspase-3, -8 or -9 cleavage, consistent with its inability to prevent apoptosis. Regarding mitochondrial activity, dissipation of mitochondrial membrane potential was significantly increased after 6 hours of Siglec-8 crosslinking compared to control cells (57%±7 versus 18%±6 loss in mitochondrial membrane potential, n=3). Cyt-c release was increased 6 hours after Siglec-8 crosslinking (15%±6) compared to control cells (2%±1) and was even higher in the presence of IL-5 (35%±13, used at concentration of 30 ng/ml). Specific inhibitors of caspase-8 and caspase-9 blocked the apoptotic effect as measured by using annexin-V staining. However, in the presence of IL-5, neither caspase inhibitor could override apoptosis induced by Siglec-8 crosslinking. In conclusion, Siglec-8-mediated eosinophil apoptosis is caspase dependent and involves loss of mitochondrial membrane potential, cyt-c release and activation of caspase-3, -8 and -9. In the presence of IL-5, Siglec-8-induced apoptosis is also regulated through mitochondria but is caspase-independent. Ligand-induced regulation via Siglec-8 may play an important role in eosinophil apoptosis in vivo, especially under conditions of cytokine priming.

(206) **CD22 Is Constitutively Associated With IgM: CD22's Ligand-Binding Domains Contribute to, But Are Not Necessary for, Association**

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CD22 is an a2,6 sialic acid binding transmembrane glycoprotein that is expressed only on the surface of mature B cells. The function of CD22 is best characterized as a negative regulator of B cell receptor signaling. Previously, we found that the ligand-binding domain of CD22 is required for function. In order to identify functional protein ligands of CD22 on the B cell surface, we biotinylated cell surface and simultaneously used DSP (dithio-bis(Succinimidyl Propionate)) to cross-link surface proteins within 12 angstroms of each other. Immunoprecipitation (IP) of CD22 from resting B cells brought down only a 75KD and a 30KD surface protein. The association of these two proteins with CD22 required the presence of the DSP crosslinker, which suggests that association is weak or transient. By reprobing western blots with specific antibodies we found that the proteins were the mu and the kappa chains of the surface IgM. Less than 2% of the total IgM co-immunoprecipitated with CD22. Reverse immunoprecipitation with anti-IgM revealed that less than 1% of total CD22 co-immunoprecipitated with IgM. CD22/IgM association did not increase after IgM activation. Phosphorylated CD22 was not preferentially associated with IgM. Deletion of the first two N-terminal Ig-like domains of CD22 decreased, but did not abolish CD22/IgM association. We also found that simply mutating the conserved arginine residues in the sialic acid binding site did not have a detectable effect on CD22/IgM association. We conclude that the Ig1 and Ig2 domains contribute to the association of CD22 with IgM but they are not necessary. A model of CD22/IgM association and function is presented.

(207) **Neither Absence of CD43, nor Deficiency of Core 2 GlcNAcT-I, Eliminate 1B11 Antibody Reactivity, the Apoptotic Phenotype, and the Reduction of CD8+ T Lymphocyte Levels in ST3Gal-I Null Mice.**

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T lymphocyte activation evokes specific changes in cell surface O-glycans. Within 48 hours of immune receptor activation, O-glycan structures on CD8+ T cells have reduced sialic acid expression on the core 1 O-glycan branch and the synthesis of a core 2 O-glycan branch has occurred. This post-activated O-glycan structure is maintained until either apoptotic death or upon differentiation into CD8+ memory T cells. We have found that the ST3Gal-I sialyltransferase is responsible for core 1 O-glycan sialylation and normally suppresses core 2 O-glycan formation. Production of this post-activated O-glycan structure thereby occurs in ST3Gal-I deficient mice. CD8+ T cell

apoptosis results in the absence of immune stimulation. These and other data have formed the basis for a model of CD8+ T cell homeostasis in the resolution of an immune response comprising a default mechanism that involves O-glycan-mediated caspase-activation. Further studies have focused on the genes responsible for induction of the post-activated O-glycan structure and the apoptotic phenotype of these CD8+ T cells. The apoptotic phenotype can be recapitulated in vitro by ligating cell surface O-glycans using 1B11 antibody or PNA lectin. We show that this apoptotic response does not involve CD43 and also continues in the absence of Core 2 GlcNAcT-I. Moreover, CD43 deficiency does not eliminate 1B11 antibody reactivity among CD8+ T cells. Similar findings were noted among Core 2 GlcNAcT-I deficient T cells. Ongoing studies are focused on identifying the glycoprotein(s) bearing the post-activated O-glycan structure and which are involved in transmitting the apoptotic signal. The continued presence of 1B11 reactivity suggests the involvement of one or more Core 2 GlcNAc transferases among the three related enzymes previously identified. Additional studies using ST3Gal-I transgenic mice are progressing in order to determine the level of Core 2 O-glycan branch participation, versus desialylation of the Core 1 O-glycan branch in apoptosis and the homeostasis of peripheral CD8+ T cells.

(208) **Rheumatoid Arthritis – Molecular Dynamics Simulations of the Fc Domain as a Function of Glycosylation**

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Rheumatoid arthritis is a disabling autoimmune disease that afflicts millions of people worldwide. In 2000, Merck reported that 2.1 million Americans suffered from the inflammation, swelling, and joint pain that characterize RA. IgG antibodies, by binding to rheumatoid factor proteins, are biological players in the disease process. A notable structural feature of IgG antibodies is the presence of *N*-linked glycans, attached to the Cγ2 domains, which extend into the core of the Fc domain. It is known that the terminal galactosyl residues of these branched glycan chains are absent in patients who display symptoms of RA. The aberrant glycosylation pattern can return to normal when RA symptoms are in remission, such as is the case in RA patients who become pregnant. Thus, there is an intriguing correlation between the glycosylation state of the Fc-glycans and the disease state. Here we examine the hypothesis that the glycosylation state of the Fc-glycans affects their mobility as well as that of the Fc domain itself. To accomplish this we have created a series of computational models of the Fc domain that differ in the number of Cγ2 domains and in the glycosylation state of the glycan chains. We have performed long timescale fully solvated molecular dynamics simulations of these models. Insight into glycan dynamics is gained from examination of the motion of the inter-glycosidic torsion angles, as well as from comparison with x-ray data. Method development, results, and preliminary interpretations are presented.

(209) **CD28 Glycosylation and Cell Death in T Cells**

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CD28 a key costimulatory molecule on T cells important for optimal T cell activation in immune responses, is a 44 Kd homodimer with five N-linked glycosylation sites. It interacts with ligands (B7, CD80 and CD86) on antigen presenting cells. Until recently, it was thought that stimulation with CD28 enhanced T cell receptor (TCR) signaling but had no effect on its own. However, recent data 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 conformational differences in cell surface CD28 influencing how the T cell responds to various stimuli. 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, was largely unaffected. By Western blot analysis using a polyclonal anti-CD28 antibody, we observed differences in CD28 molecular weight (Mr) 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 Mr (50-60Kd). To determine if the observed differences in Mr were due to differential glycosylation, we used glycosidases followed by Western blotting. CD28 from Jurkat was digested by N-glycanase F (PNGaseF), but not by endoglycosidase H (endoH), whereas CD28 from H9 cells was digested by both glycosidases. 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 Golgi. To study whether glycosylation was important for the biological activity of CD28, we examined the effect of tunicamycin (an inhibitor of N-linked glycosylation) on cell surface CD28 expression. Treatment of Jurkat T cells with tunicamycin for 4-h resulted in death of about 20% of the cells. Levels of cell surface CD28 were also reduced by about 30%. The same cell population that bound AnnexinV was also CD28-negative. However, the remaining AnnexinV negative cells expressed high levels of CD28. By contrast, H9 T cells did not increase AnnexinV binding nor change CD28 expression after tunicamycin treatment. To test whether tunicamycin affected the biological response to ANC28, we treated for 4-h, washed with PBS and then exposed Jurkat T cells to ANC28. After tunicamycin treatment, Jurkat T cells were no longer susceptible to ANC28 killing and CD69 upregulation was reduced about 70%. These data suggest that the five N-linked glycosylation sites on CD28 are important for optimal T cell activation and survival. Further, differential glycosylation may regulate cell death in T cells by changing the conformation of CD28 on the cell surface.

(210) **Altered Branching Patterns of Mammalian N-Glycans Result in Autoimmune Disease**

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Autoimmune disease is a prevalent human disorder affecting an estimated 5% of the world's population. Although various mechanisms are postulated to be involved in disease occurrence, the underlying genetic and environmental factors remain largely undefined. We have previously shown that a genetic mutation which alters endogenous protein glycosylation results in an autoimmune syndrome in mice that is similar to human systemic lupus erythematosus (SLE). This defect disrupts α-mannosidase II (aM-II), a Golgi-resident enzyme that processes the branching pattern of N-glycans. aM-II produces a hybrid branch pattern that is a precursor to complex type N-glycan formation. We have shown that the absence of aM-II results in glomerular deposition of complement component C3 and immunoglobulin, development of glomerulonephritis, hematuria, proteinuria, and in some cases end-stage kidney disease resulting in death. Anti-nuclear antibodies are detected, along with auto-antibodies that react against endogenous proteins of various tissues -- including those bearing altered N-glycans. Furthermore, we have found that aM-II deficiency results in a reduction of serum mannose binding lectin (MBL) levels, and that MBL-A and MBL-C binding is increased towards the N-glycan structures present in the aM-II deficiency. Finally, we find that glomerular deposition of MBL is also significantly increased in aM-II deficient animals. The role of complement activation and other specific immune components are currently under investigation as possible causes of this SLE-like syndrome. Although the possibility of widespread activation of the innate immune system remains, it appears unlikely given the results obtained upon measurements of interleukin-6 and TNF-a, which indicate normal circulation levels for both.

(211) **Deficiency of Heparan Sulfate N-deacetylase/N-sulfotransferase-1 in Endothelium Impairs Inflammation**

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Heparin and heparan sulfate (HS) interacts with various components of inflammatory cascade in vitro, such as P- and L-selectins, inflammatory mediators, and chemokines. To study the significance of these interactions in vivo, glucosaminyl N-deacetylase/N-sulfotransferase-1 (NDST1) was knocked out in endothelial cells, T-cells and B-cells using the Cre-loxP system. NDST1 deficiency resulted in a structural alteration of heparan sulfate as revealed by reduction in staining with heparan sulfate probes and by direct measurement of glucosamine N-sulfate content. The reduction was not complete due to the expression of NDST2, a second isozyme expressed ubiquitously in cells. The NDST1 deficient mice showed impaired inflammatory responses in multiple types of assays. The inflammatory defect was not due to the enzyme deficiency in leukocytes, but specifically reflected changes in the endothelium. No other changes in hemostasis occurred in the mice. To our knowledge this is the first direct demonstration for a role of endothelial heparan sulfate in inflammatory reactions.

(212) **N-Acetylglucosamine-6-O-sulfotransferase-1 and -2 Cooperatively Control Lymphocyte Homing through an Essential Role in Sulfation of L-Selectin Ligand Oligosaccharides in High Endothelial Venules**

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Lymphocytes encounter antigens derived from foreign pathogens and initiate immune responses in lymph nodes. Thus lymphocyte homing to lymph nodes is essential for appropriate immune responses. Lymphocyte homing is controlled by a specific interaction between lymphocyte homing receptor L-selectin and its glycoprotein ligands, such as GlyCAM-1 and CD34, expressed on high endothelial venules (HEV) in lymph nodes. Previous studies in our laboratory have revealed that these glycoproteins are modified with O-glycans containing 6-sulfo sialyl Lewis x in either core 2 or extended core 1 branch, or in both of the branches (Yeh et al., Cell 105, 957-969, 2001). The same studies have revealed that MECA-79 antibody binds to O-glycans containing 6-sulfo N-acetylglucosamine in the extended core1 structure.

In order to determine the roles of O-glycans containing 6-sulfo sialyl Lewis x, we generated gene mutant mice deficient in the HEV-restricted sulfotransferase, N-acetylglucosamine-6-O-sulfotransferase-2 (GlcNAc6ST-2, HEC-GlcNAc6ST/LSST). In GlcNAc-6ST-2-deficient mice, binding of MECA-79 antibody to lymph node HEV was almost abolished, except for the binding observed in the abluminal lining of HEV, suggesting that N-acetylglucosamine (GlcNAc)-6-O-sulfation in extended core 1 branch is mediated mainly by GlcNAc6ST-2. We have also carried out oligosaccharide structural analysis of GlyCAM-1 isolated from wild-type and GlcNAc6ST-2-deficient mice. As expected, GlcNAc-6-O-sulfation in extended core 1 branch in GlcNAc6ST-2-deficient mice was significantly reduced to 10 to 20% of the wild-type mice levels. However, approximately 50% of GlcNAc-6-O-sulfation in core 2 branch of O-glycans attached to GlyCAM-1 still remained in GlcNAc6ST-2-deficient mice. Consistently, only a partial reduction in lymphocyte homing was observed in GlcNAc6ST-2-deficient mice.

In mice, four members of GlcNAc-6-O-sulfotransferases have been reported. One of the family members, GlcNAc6ST-1 is widely expressed in various tissues including lymph node HEV. We thus crossbred GlcNAc6ST-2-deficient mice with recently generated GlcNAc6ST-1-deficient mice in order to determine if the remaining GlcNAc-6-O-sulfation of L-selectin ligand oligosaccharides is synthesized by GlcNAc6ST-1. While GlcNAc6ST-1 and GlcNAc6ST-2 singly deficient mice showed approximately 20 % and 50% reduction in lymphocyte homing, respectively, GlcNAc6ST-1 and -2 doubly deficient mice showed approximately 75% reduction in lymphocyte homing. Immunofluorescence studies revealed that binding of MECA-79 antibody to lymph node HEV of the doubly deficient mice was completely abolished, suggesting that GlcNAc-6-O-sulfation in extended core 1 branch of O-glycans in HEV is absent in the doubly deficient mice. We are in the process of analyzing structures of O-glycans attached to GlyCAM-1 to determine if GlcNAc-6-O-sulfation of both extended core 1 and core 2 branchs of O-glycans is completely diminished in the doubly deficient mice.

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(213) **Natural Killer Cell Receptor, Siglec-7, Overexpressed on U937 Cells Binds to α2,8-Disialo-Expressing Cells**

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Siglec-7 is a sialic acid-binding lectin recently identified as an inhibitory receptor on natural killer cells and monocytes. We and other investigators have characterized sugar-binding specificity of Siglec-7, and found that the lectin preferably binds a unique oligosaccharide structures such as α2,8-disialyl residue (sialyl α2,8-sialyl) and branched α2,6-sialyl residue (galactosyl β1,3-[sialyl α2,6]-hexosaminyl) [1, 2]. Here we examine whether Siglec-7 on the cells recognizes the unique oligosaccharide structures that are expressed on potential target cells. Siglec-7 cDNA was transfected to monocytic cell line, U937 cells, to isolate stable transformants (U937-Siglec-7). The expression of the lectin was confirmed by anti-Siglec-7 monoclonal antibody and its sugar-binding activity was detected with α2,8-disialyl-conjugated polyacrylamide probe by flowcytometry. Human erythroleukemic cell line, K562 cells, as potential target cells were transfected with a sialyltransferase, ST8Sia VI, which synthesized α2,8-disialyl residue on O-glycans (K-562-disialo) [3]. The expression of α2,8-disialyl residue was confirmed by anti-disialyl residue antibody, and the expression was completely abolished by the addition of benzyl N-acetylgalactosamine, a potent inhibitor for O-glycan biosynthesis. U937-Siglec-7 cells strongly bind to K-562-disialo cells but not to control cells. In addition, the binding induced tyrosine-phosphorylation of the lectin, suggesting that Siglec-7 on the cell can bind to α2,8disialyl residue on the target cells. We will also present data on Siglec-7-dependent cytosolic signaling, in which phospho-tyrosine residues of the lectin recruit phosphatases (SHP-1 and -2) to modify cellular functions such as calcium mobilozation. References 1. T. Yamaji, et al. (2002) A small region of the natural killer cell receptor, Siglec-7, is responsible for the unique specificity for a2,8disialyl and branched a2,6sialyl residues: a comparison with Siglec-9. J. Biol. Chem., 277, 6324-6332: 2. T. Yamaji, et al. (2003) Characterization of sugar-binding specificity of Siglec family proteins. in Methods in Enzymol., "Carbohydrate recognition in biological systems " edited by Y.C. Lee and R. Lee, Academic Press, in press 3. S. Takashima, et al. (2002) Molecular cloning and expression of a sixth type of α2,8-sialyltransferase (ST8Sia VI) that sialylates O-glycans. J. Biol. Chem., 277, 24030-24038

(214) **Contributions of Ca^{2+} to Galectin-1-Induced Exposure of Phosphatidylserine on Activated Neutrophils**

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Apoptotic cells gradually redistribute phosphatidylserine (PS) to the cell surface by both Ca^{2+}-dependent and -independent mechanisms. Binding of dimeric galectin-1 (dGal-1) to glycoconjugates on fMLP-activated neutrophils exposes PS and facilitates neutrophil phagocytosis by macrophages, yet does not initiate apoptosis. We asked whether dGal-1 initiated Ca^{2+} fluxes that were required to redistribute PS to the surface of activated neutrophils. Like fMLP, dGal-1 rapidly elevated cytoplasmic Ca^{2+} levels in Fluo-4-loaded neutrophils. An initial Ca^{2+} rise from intracellular stores was followed by movement of extracellular Ca^{2+} to the cytosolic compartment, with return to basal Ca^{2+} levels within 10 min. Prolonged occupancy of dGal-1 was required to maximally mobilize PS on the surface of fMLP-activated neutrophils. Chelation of extracellular Ca^{2+} did not prevent PS mobilization. Chelation of intracellular Ca^{2+} revealed that that both fMLP and dGal-1 must transiently release Ca^{2+} from intracellular stores to optimally redistribute PS. Neutrophil pretreatment with genistein, a tyrosine kinase inhibitor, or with wortmannin, a phosphatidylinositol 3-kinase inhibitor, blocked Ca^{2+} mobilization by dGal-1 but not by fMLP. Genistein or wortmannin also partially inhibited the dGal-1-induced redistribution of PS to the surface of fMLP-activated neutrophils. Ca^{2+} mobilization by ionomycin did not permit dGal-1 to mobilize PS, indicating that the G-protein-coupled fMLP receptor initiated both Ca^{2+}-dependent and -independent signals that facilitated dGal-1-induced exposure of PS. These results demonstrate that transient Ca^{2+} fluxes contribute to a sustained redistribution of PS on activated neutrophils treated with dGal-1.

(215) **Changes in Glycosylation-Specific Gene Expression During Memory T Cell Differentiation**

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The generation of a specific CD8 T cell immune response following viral infection is critical for host defense. Following viral control and apoptosis of up to 90% of antigen-specific T cells, a subset of long-lived memory T cells remain which provide protection against re-infection and disease. Activation of naïve T cells and their differentiation to memory cells is accompanied by dramatic changes in gene transcription, protein expression, and post-translational modifications of proteins. To further characterize the changes in glycosylation during memory T cell differentiation we have performed a systematic examination of differential glycosyltransferase and carbohydrate binding protein gene expression among in vivo generated antigen-specific T cell populations using the Glycochip. This analysis has revealed changes in gene expression that correlate with glycophenotypic changes among naïve,

effector, and memory T cell populations. The differences in cell surface glycosylation among these T cell populations may yield insights into memory T cell functions including T cell trafficking and homeostatic maintenance.

(216) Sialic Acids and CD22 Function: Exploring the Effects on Interactions with CD45 and sIgM, Constitutive Endocytosis, and Antibody-Induced Endocytosis

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CD22/Siglec-2 is a B cell-specific molecule that regulates surface IgM (sIgM)-generated signaling responses via cytosolic tyrosine–based motifs. CD22 is also an I-type lectin that specifically recognizes alpha 2-6-linked sialic acid (Sia) via an extracellular amino-terminal V-set Ig-like domain. For unknown reasons, this extracellular sialic acid-based interaction is required for optimal cytosolic signaling responses. In resting mature B cells, the CD22 Sia-binding site is mostly masked by interactions with as yet unknown sialylated ligands. Several molecules that bear alpha 2-6 sialylated ligands e.g., cell surface CD45 or sIgM, as well as circulating molecules such as IgM and haptoglobin, have been suggested as candidate ligands. These have been suggested mostly based on the ability of recombinant soluble CD22 to bind them in a sialic acid dependent manner in vitro. However, the dimeric CD22 chimeras used for such studies can interact with any molecule that has a high density of alpha 2-6-linked sialic acids, especially when the chimera is immobilized on beads. It has nevertheless been proposed that CD22 associates with CD45 and sIgM primarily via sialic acid dependent interactions. However, coimmunoprecipitation using "mild detergent" approaches have reported interactions only at levels of a few percent, and a role of sialic acid recognition has not been directly shown. Since the single-site binding affinity of CD22 to alpha 2-6-linked sialic acids is relatively poor, it cannot survive the repeated washing involved in standard immunoprecipitation protocols. Surface labeling followed by cross-linking thus provided the best hope of quantitatively detecting native interactions. However, labeling and cross-linking are typically carried out in a step-wise fashion, and there is a serious risk of perturbing the critical interactions during the first labeling step, prior to cross-linking. The same problems apply if manipulations such as sialidase treatment are used before cross-linking is done.

Here we use a novel approach for simultaneous biotinylation and cross-linking of cell-surface molecules at 4°C to show that CD22 actually associates with CD45 and sIgM at substantially higher levels than previously reported using other methods. The data also suggest the possibility of cell surface multimers of CD22. Transfected human CD22 associated with similar partners in mouse B cells, indicating that these interactions are evolutionarily conserved. However, mutation of a CD22 arginine residue required for Sia recognition does not substantially affect these protein:protein interactions. Abrogating Sia interactions also does not affect the constitutive endocytosis of CD22. In contrast, Sia removal does enhance the much faster rate of antibody-mediated endocytosis, as well as killing by an anti-CD22 immunotoxin currently in use in clinical trials for lymphoma treatment. In contrast to the unstimulated state, sIgM ligation inhibits both antibody-induced endocytosis and immunotoxin killing. Thus, the signal modulating activity of CD22 Sia recognition cannot be explained by mediation of selective interactions with specific cell surface molecules, nor by effects on constitutive turn over of the molecule via endocytosis. However, the effects on antibody-mediated endocytosis could be of relevance in the immunotoxin treatment of lymphomas.

(217) Altered Leukocyte Maturation in *Schistosoma mansoni*-Infected Galectin-3 Deficient Mice.

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Galectin-3, a β-galactoside binding animal lectin, plays a role in cell-cell and cell-matrix interactions. Extracellular galectin-3 modulates cell migration and adhesion in several physiological and pathological processes. Intracellular galectin-3 may interfere with proliferation and gene expression through its interaction with transcription factors, e.g. Galectin-3 is highly expressed in activated macrophages. Here we have investigated both wild type and galectin-3 deficient mice, which were infected with *Schistosoma mansoni*. *S. mansoni* elicits a well characterized macrophage-dependent granulomatous inflammatory reaction. Cells from bone marrow, spleen, mesenteric ganglia, peritoneal cavity and from the granuloma were harvested and had their phenotype analyzed. Remarkable changes were observed both in the differentiation process of monocytes and in B lymphopoiesis. While in wild type animals, the monocyte to macrophage ratio within granulomas indicated a clear accumulation of the more differentiated cells, this ratio was inverted in galectin-3 deficient mice. Therefore, galectin-3 seems necessary for the completeness of myeloid differentiation within the granuloma. B cells also express galectin-3 on the cell surface. Around 85% of $B220^{high}$ lymphocytes were also positive for galectin-3. When cells from the peritoneal cavity were analyzed, 90% of the $B220^{low}/CD5^{+}$ cells (B1 lymphocytes) also expressed galectin-3. *S. mansoni*-infected galectin-3 deficient mice had a relative B-lymphopenia, as compared to wild type animals. Interestingly, there was a clear increase in the number of plasma cells in spleen and in the mesenteric ganglia. This increase in plasma cells was associated with higher concentrations of plasma IgG and IgE. Long term cultures of bone marrow derived stromal cells under myelopoietic conditions (Dexter protocol) and lymphopoietic conditions (Whitlock-Witte protocol) were then performed. No differences were observed between both wild type and galectin-3 deficient mice using the Whitlock-Witte protocol. However, following the Dexter protocol, no lipid accumulating stromal cells were found in galectin-3 deficient mice. These latter cells are essential to proper myeloid differentiation. Galectin-3 seems to be associated with delayed maturation of macrophages and accelerated differentiation of B cells to plasma cells.

(218) The Population Genetic History of Siglec-L1: Implications for the Human-Specific Loss of Neu5Gc.

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Sialic acids are 9-carbon sugars that are major components of cell surface and secreted glycoconjugates in deuterostomes. Two common sialic acids in mammals are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc), which differ by a hydroxyl group. Most mammalian cell surfaces display both Neu5Ac and Neu5Gc. However, humans are genetically unable to synthesize Neu5Gc due to the inactivation of the gene encoding the CMP-N-acetylneuraminic acid hydroxylase (CMAH) (1). This deleted form of the CMAH gene arose after the common ancestor of Homo and Pan and fixed in the human lineage. The loss of the CMAH hydroxylase resulted in a loss of Neu5Gc and a corresponding excess in Neu5Ac expression compared to other mammals. This change in human sialic acid composition likely had major functional consequences for endogenous and exogenous cell recognition by sialic acid-binding receptors like the Siglecs. The Siglecs (sialic acid binding Ig-like lectins) are mammalian cell-surface type 1 transmembrane proteins comprised of an amino terminal Ig V-set domain, variable numbers of Ig C2-set domains, a single-pass transmembrane domain, and a cytoplasmic tail containing tyrosine-based signaling motifs. Sialic acid recognition occurs in the V-set domain and is partly mediated through a conserved arginine residue that forms a salt bridge with the sialic acid carboxylate group. A change in sialic acid expression could thus exert an influence on the evolution of V-set domains. We are investigating the evolutionary history and functional significance of Siglec-L1 (Siglec-Like-1) in order to better understand the implication of the human-specific loss of Neu5Gc. Siglec-L1 contains 2 adjacent V-set domains, unique among the Siglecs. In humans, both of these domains are mutated at the critical arginine residue and are thus unable to bind sialic acids. Restoration of the arginine binding site in human Siglec-L1 receptor results in a strong preference for Neu5Gc over Neu5Ac (2); this suggests that the knockout of sialic acid binding may have been a response to the change in the human sialic acid phenotype. In that study (2) we also noted the occurrence of a polymorphic frameshift mutation in the first V-set domain of some individuals that appears to eliminate the nonfunctional protein from cell surfaces altogether. Here we investigate the frequency of the frameshift mutation and other polymorphic sites in the Siglec-L1 Vset-1 domain in order to study its evolutionary history and implications for human sialic acid biology. We find that the frameshift mutation is carried by ~60% of human alleles and that another 10% of alleles carry nonsense mutations, meaning that ~70% of human alleles are incapable of producing an intact Siglec-L1 receptor. This suggests that loss of Siglec-L1 is not deleterious. Siglec-L1 may reflect neutral evolution at an effectively pseudogenized (i.e. non sialic acid binding) gene or, alternatively, the selective elimination of a malfunctioning (i.e. non sialic acid-binding) cell surface receptor. Population genetic and molecular evolutionary characterization of adjacent exonic domains will clarify the mode of evolution in the Siglec-L1 Vset-1 domain.

(1) H.-H. Chou et al. 1998 A mutation in human CMP-sialic acid hydroxylase occurred after the *Homo-Pan* divergence. PNAS 95: 11751 - 11756.

(2) T. Angata et al. 2002 A second uniquely human mutation affecting sialic acid biology. JBC 276: 40282 - 40287.

(219) **Identification of Ligands for Galectin-10 (Charcot-Leyden Crystal Protein) in Human Eosinophils: Selective Interaction with Granule Cationic Ribonucleases**

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Human eosinophil Charcot-Leyden Crystal (CLC) protein, which forms the distinctive hexagonal bipyramidal crystals that are a hallmark of eosinophil participation in allergic and other inflammatory reactions, was originally identified as eosinophil lysophospholipase (LPLase). However, our recent work has shown that CLC protein is not eosinophil LPLase, rather it belongs to the galectin superfamily of animal lectins, and is now referred to as Galectin-10 (Gal-10). Its glycoconjugate-containing ligand(s), cellular function and role in eosinophil biology and allergic diseases have not been determined. To identify biologically relevant intracellular ligand(s) for Gal-10, we performed Gal-10 ligand blotting in combination with glycosidase (PNGase F, which cleaves N-linked sugars from glycoproteins) digestion of eosinophil whole cell lysates. A 21 kD protein band in eosinophil whole cell lysate was identified, which was also positive by modified PAS glycoprotein staining. The MALDI/MS analysis of this putative Gal-10 ligand showed that it was comprised of the two eosinophil granule cationic ribonucleases, eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP), both of which are highly glycosylated with N- and O-linked sugars. Gal-10 ligand blotting of the purified eosinophil granule cationic proteins, EDN, ECP, and MBP (major basic protein), confirmed the selective binding of Gal-10 to EDN and ECP, but not MBP, one of the most cationic granule proteins, eliminating binding due simply to charge interactions. N-linked sugars are not involved in the Gal-10 and EDN/ECP interaction, since digestion of either whole cell lysate of human eosinophils or purified granule proteins with PNGase F did not diminish the intensity of Gal-10 binding detected by ligand blotting. Co-immunoprecipitation of blood eosinophil whole cell lysate using anti-Gal-10 antibody showed that EDN/ECP co-immunoprecipitate with Gal-10. Purification of Gal-10 from whole cell lysates of both an eosinophil cell line (AML14.3D10) and blood eosinophils using an affinity purified anti-Gal-10 antibody column showed that EDN/ECP co-purify with Gal-10, whereas MBP does not. Gal-10 also binds to murine eosinophil-associated ribonucleases (EARS) by ligand blotting, despite the absence of a Gal-10 ortholog in the mouse eosinophil. In vivo studies using immunofluorescence confocal microscopy show that interleukin-5 activation of blood eosinophils induces the cellular co-localization of Gal-10 and EDN. Our results demonstrate that Gal-10 selectively interacts with the two human eosinophil ribonucleases, EDN (RNS2) and ECP (RNS3), but not another highly charged granule cationic protein, MBP. The binding of Gal-10 to EDN/ECP is likely through O-linked sugars and/or the core polypeptides. Of note, Gal-10 does not likely function as a ribonuclease inhibitor, since CLC-derived Gal-10 failed to inhibit the ribonuclease activities of EDN, RNase A or RNase B. Thus, Gal-10 may function as a carrier for the vesicular transport of EDN/ECP to sequester their ribonuclease activities during eosinophil activation and secretion. Supported by NIH Grants F32 AI51137 (to LL) and T32 DK07739 and AI25230 (to SJA).

(220) **Human Galectin-1 Binds with High Affinity to α2,3-sialylated and Nonsialylated Glycans Containing Poly-N-acetyllactosamine**

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Galectin-1 (Gal-1) is a member of the galectin family of glycan binding proteins and occurs as a ~ 29.5 kD noncovalent dimer that is widely expressed in many tissues. The endogenous ligands for Gal-1 are not well understood, but there is evidence that Gal-1 may recognize glycoconjugates containing terminal N-acetyllactosamine (LN) (Galβ3GlcNAc-R) and poly-N-acetyllactosamine (PL) (-Galβ3GlcNAcβ-)$_n$ structures. Here we report our studies on the binding specificity of human Gal-1 towards a wide variety of neutral and α3-sialylated glycans in both a fluorescence-based solid phase binding assay and in equilibrium gel filtration. Gal-1 bound preferentially and with high affinity (apparent K_d ~ 1.7 μM) to immobilized, long chain PL presented either on glycopeptides within a core-2 based O-glycan or as linear, peptide-free glycans. Gal-1 binding affinity was proportional to the number of LN repeats (LN3>LN2>LN). Binding to immobilized, single LN-containing structures was undetectable. Although terminal Gal residues were important for Gal-1, Gal-1 bound similarly to α3-sialylated glycan versus their non-sialylated derivatives. Unexpectedly, Gal-1 preferentially recognized extended PL only when ligands were surface-bound. Gal-1 binding to ligands free in solution, as in equilibrium gel filtration, was relatively low affinity and displayed no preference to PL-containing glycans. To explore the binding of Gal-1 to cell surface, we measured Gal-1 binding to native and desialylated HL-60 cells. Gal-1 bound to both native and desialylated HL-60 cells with similar affinity as observed toward immobilized LN3. Treatment of HL-60 cells with endo-β-galactosidase, which cleaves PL sequences, reduced binding by Gal-1, indicating the surface PL-containing glycans were ligands. These results demonstrate that Gal-1 preferentially recognizes extended PL when glycans are surface bound as expressed on cells.

(221) **Sialoside Regulation of B Cell Function through CD22**

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CD22, a siglec family member, is a negative regulator of B cell function. It is generally accepted that recruitment of CD22 to the B cell receptor complex (BCR), and its subsequent recruitment of the phosphatase SHP-1 down regulates B cell signaling. In support of this, mice deficient in CD22 exhibit a hyperimmune phenoytpe including increased calcium flux in response to BCR crosslinking, and increased antibody production (1), including anti-DNA antibodies (2). As a siglec family member, CD22 binds to sialic acid containing glycans. In particular it exhibits high specificity for the sequence Siaα2,6Gal, which is expressed *in vivo* by the enzyme ST6Gal I. Surprisingly, mice deficient in this enzyme, and thus the ligand for CD22, are immuno-suppressed, exhibiting decreased calcium flux and suppressed antibody production to T dependent or independent antigens (3).

To further investigate the phenotypes of the CD22 and ST6Gal I null mice in regulation of the immune response we have generated double null mice deficient in both CD22 and ST6Gal I. The resulting mice are viable and fertile and exhibit normal development. We have compared the double nulls to each of the single nulls and the wild type mice to delineate any alterations in the lymphocyte populations or B cell activation. There are no significant changes in CD4+, CD8+ T cells, or B cells populations. However, relative to wild type, the double nulls, similar to ST6Gal I single nulls, did have reduced levels of surface IgM (40-50%) and CD180 (30-40%).

As observed previously, following activation, B cells from CD22 null mice gave slightly higher or similar levels of proliferation relative to WT, while proliferation of cells from ST6Gal I single nulls were dramatically suppressed. By comparison, B cells from the double null mice gave levels of proliferation similar to those of WT mice. These data suggest that at least part of the immuno-compromised phenotype of the ST6Gal I null mice can be abrogated by eliminating CD22. (Supported in part by GM25042 to B.E.C., GM60938 to J.C.P., and P01-HL57345 to J.D.M)

(1) Nitschke *et al.* (1997) Current Biology, **7**, 133
(2) O'Keefe *et al.* (1999) J. Exp. Med., **189**, 1307
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(222) **Alpha- and Theta-Defensins Are Miniature Lectins**

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Human neutrophils, NK cells and CD8+ T-cells contain 3.5 kDa, cysteine rich peptides called alpha-defensins (HNPs). These 3.5 kDa molecules have broad-spectrum antimicrobial properties, attract T-lymphocytes and other mononuclear cells in vitro and in vivo, enhance wound healing, and are immunostimulatory. In addition to alpha-defensins, the leukocytes of non-human primates also contain 2 kDa cyclic octadecapeptides called theta-defensins. Theta defensin genes arose via mutation of pre-existing alpha defensin genes. A subsequent mutation that is shared by humans, gorillas and chimpanzees converted them into expressed pseudogenes. Both *f*nalpha defensins (Zhang, et al., Science, 298: 995, 2002) and theta defensins (Cole, A.M., ET al., PNAS, 99:1813, 2002) protect otherwise susceptible cells from infection by HIV-1 in vitro. We recently reported a) that theta-defensins are lectins; b) that they bind viral and cell surface glycoproteins involved in virus

entry with high affinity; and c) that their binding properties and antiviral activity are correlated (Wang, W. et al., J. Immunol. 170: 4708, 2003). Thus, theta defensins have the dual distinctions of being the only cyclic peptides of animal origin and the smallest lectins described to date. Because of their common ancestry and similar antiviral properties, we wondered if human alpha defensins were also lectins, and tested their ability to bind various glycoproteins and neoglycoproteins by surface plasmon resonance (SPR). HNP-2 bound gp120 with a Kd of 15.8 nM, and it bound CD4 even higher affinity (Kd, 8.0 nM). When we treated immobilized gp120 with endo- and/or exoglycosidases, the ability of HNP-2 to bind gp120 was reduced or abrogated. HNP-1 bound gp120 and CD4 almost as well as HNP-2 and HNP-3 bound them with somewhat lower affinity (Kd 52.8 nM for gp120, and 34.9 nM for CD4). However, HNP-4, the most cationic human leukocyte alpha-defensin, bound gp120 with a Kd of 214 nM. Since HNP-4 bound equally well to native and deglycosylated gp120, it did not appear to bind carbohydrate moieties of the viral glycoprotein. We performed additional SPR studies with a panel of over 40 different neoglycoproteins that were synthesized and kindly provided by Professor H-J. Gabius (Veterinary Medicine Faculty, Ludwig-Maximilians University, Munich). These confirmed that HNPs 1-3 were lectins and provided valuable information about their sugar-specificity. Defensin peptides are components of the innate immune system that contribute to host defense. The ability of alpha and theta defensins to recognize and bind sugars plays a central role in their antiviral activities; and may also contribute to their signaling functions and ability to amplify adaptive immune responses. The small size (18 residues) of theta-defensins makes them attractive prototypes for a variety of uses- not the least of which would be as topical microbicides to prevent sexual transmission of HIV-1 infection.

(223) α1,2-Linked Fucosyloligosaccharides Comprise A Major Component of the Innate Immune System of Human Milk

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Breastfed infants have less morbidity and mortality due to diarrhea than non-breastfed infants. This protection is often attributed primarily to the secretory antibodies found in human milk, products of the acquired immune system. However, we have found that the oligosaccharides of human milk strongly inhibit several agents that produce diarrhea. To test the hypothesis that human milk fucosyloligosaccharides are part of an innate immune system, we addressed whether their expression 1) depends on maternal genotype and 2) protects breastfed infants from pathogens. Thus, the relationship between maternal Lewis blood group type and milk oligosaccharide expression, and between variable oligosaccharide expression and risk of diarrhea in their infants, was studied in a cohort of 93 Mexican breastfeeding mother-infant pairs. Oligosaccharides were isolated from each milk sample and perbenzoylated, whereupon they were resolved and measured by reversed-phase HPLC. Milk of the 67 Le^{a-b+} mothers contained more LNF-II (Le^a) and 3-FL (Le^x) (oligosaccharides whose fucose is exclusively α1,3- or α1,4-linked) than milk from the 24 Le^{a-b-} mothers; milk from Le^{a-b-} mothers contained more LNF-I (H-1) and 2'-FL (H-2), whose fucose is exclusively α1,2-linked. The pattern of oligosaccharides varied among milk samples; in each milk sample, the pattern was summarized as a ratio of 2-linked to non-2-linked fucosyloligosaccharides. Milks with the highest ratios were produced primarily by Le^{a-b-} mothers; those with the lowest ratios were produced exclusively by Le^{a-b+} mothers ($P<0.001$). Thus, maternal genetic polymorphisms expressed as Lewis blood group types are expressed in milk as varied fucosyloligosaccharide ratios. Stable toxin (ST) of *Escherichia coli*, a common cause of diarrhea in infants, is inhibited *in vivo* by α1,2-linked fucosyloligosaccharides of human milk. To test the clinical significance of this human milk fucosyloligosaccharide, we investigated the relationship between variation in expression of α1,2-linked fucosyloligosaccharides in individual milk samples to the risk of ST-associated diarrhea in infants consuming this milk. The four infants who developed ST-associated diarrhea were consuming milk with lower ratios (4.4 ± 0.8 [SE]) than the remaining infants (8.5 ± 0.8; $P<0.001$). However, diarrhea associated with ST-*E. coli* accounts for less than 2% of the total diarrhea in this population. More common enteric pathogens include campylobacter and rotaviruses, whose intestinal receptors contain α1,2-linked fucosyl moieties. Thus, we investigated whether 2-linked fucosylated oligosaccharides in milk were also related to the nursing infant's risk of diarrhea due to pathogens other than ST. The 27 infants who developed moderate-to-severe diarrhea of any cause were consuming milk with lower ratios (6.1 ± 0.9) than the 26 who remained healthy (10.5 ± 1.9, $P=0.042$). Thus, milk with higher 2-linked to non-2-linked fucosyloligosaccharide ratios affords greater protection against infant diarrhea. We conclude that specific oligosaccharides comprise a major element of an innate immune system of human milk.

(224) Is the Expression of Human Milk Fucosylated Oligosaccharide during the First Month of Lactation Representative of the First Year of Lactation?

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Human milk oligosaccharide expression varies between mothers and over the course of lactation. The relationship between variation in human milk oligosaccharide expression and infant health is a topic of current interest. Studies relating human milk composition to infant health outcomes would be expedited if collection and analysis of milk samples in the first month of lactation could represent milks throughout lactation. However, it has not been established whether oligosaccharide measurements in the first month of lactation are correlated with oligosaccharide values over the course of lactation. To address this question, we analyzed 80 milk samples collected from 11 subjects between week 1 and month 12 postpartum, and determined the correlation between the oligosaccharide values of milk samples collected during the first month compared with milk samples collected between months 3–12 postpartum. Oligosaccharides were isolated from each milk sample and perbenzoylated, whereupon they were resolved and quantified by reversed-phase HPLC. All study mothers were secretors. Oligosaccharide values for week 1 (colostrum) samples did not correlate well with later lactation; however, oligosaccharide values from samples collected during weeks 2–4 tended to be highly correlated with one another and with values from samples later in lactation. The highest correlations were found for summary measures of α1,2-linked fucosylated oligosaccharide content of human milk, i.e., the ratio of 2- to non-2-linked fucosyloligosaccharide [$r=0.96$, $P<0.001$] and 2-linked fucosylated oligosaccharide as a percentage of total oligosaccharides measured [$r=0.81$, $P=0.003$]. Values for the most abundant 2-linked fucosylated oligosaccharide, 2'-fucosyllactose, when expressed as a percentage of total oligosaccharide, were also highly correlated between weeks 2–4 and months 3–12 ($r=0.87$, $P<0.001$). Values of other individual fucosylated oligosaccharides were correlated between weeks 2–4 and months 3–12 (r values ranging from 0.66 to 0.77, $P<0.05$), but no correlation was observed for the two major fucosylated oligosaccharides lacto-*N*-fucopentaose-I and lactodifucotetraose. We conclude that oligosaccharide patterns of expression in milk during the 2nd through 4th week of lactation are strongly representative of the patterns from milk produced during months 3–12 of lactation for many, but not all, of the oligosaccharides measured. These results suggest a common genetic basis for variation in expression of some oligosaccharides throughout lactation, and that oligosaccharide expression over the course of lactation is controlled in a coordinated fashion. Furthermore, mothers can be classified as high or low expressors of specific types of oligosaccharides on the basis of a measure early in lactation; this early measure is valid for comparing variation in milk oligosaccharide expression throughout lactation to clinical consequences for her nursing infant.

(225) Investigating the Regulation and Function of Polysialic Acid on Natural Killer Cells

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Cell surface expression of CD56 (NCAM) demarcates two subsets of human lymphocytes with natural killing ability: natural killer cells (NK cells) defined as CD56+, CD3-; and killer T cells (NKT cells), defined as CD56+, CD3+. This protein is the primary scaffold for the glycan known as polysialic acid (PSA). Experimental evidence suggests that the addition of PSA, a bulky and negatively-charged glycan, to NCAM abrogates this molecule's ability to participate in homophilic or heterophilic binding (Fujimoto et al. 2001). The nervous system appears to exploit this modulation of NCAM binding by regulating PSA levels according to the need for plasticity (during development) or stability (in adulthood) (Eckhardt at al.). Although expression of CD56 has long been used as a marker for NK and NKT cells, both the presence and the role of PSA on these cells has been largely ignored. Here, we explore the regulation and function of PSA on these lymphocytes.

Using flow cytometry, we found that PSA was expressed at low levels on circulating NK and NKT cells, and that expression was increased after in vitro

stimulation with IL-2. These observations led us to investigate the regulation of PSA expression, beginning with the transcription of the enzyme that produces PSA in leukocytes, the polysialyltransferase ST8SiaIV (PST). Previously published Northern blots suggested that leukocytes might generate different spliceforms of PST from those expressed by the nervous system (Angata et al.). RACE PCR was performed to identify the 5' and 3' ends of the leukocyte PST transcripts. Surprisingly, cloning and sequencing of these RACE PCR products revealed multiple splice variants. While the 5' end was the same as previously published sequences, the 3' end displayed considerable heterogeneity. Although we did find sequences corresponding to the two previously described transcript variants, our assay also identified at least six novel transcripts. Interestingly, many of these transcripts included sequences from regions of the PST gene that were previously considered to be introns. The significance of these novel transcripts has not yet been addressed, and we do not yet know whether they are translated into protein.

Currently, we are using several comparative approaches to assess the functional role of PSA on NK cells. These experiments all rely on the ability to selectively remove PSA from cell surfaces using the enzyme endoneuraminidase N. With confocal microscopy, we are visualizing the localization of CD56/PSA in NK cell/target conjugates involving both cytotoxic and non-cytotoxic synapses. Flow cytometry-based killing assays compliment the microscopy, allowing us to look for impairment or enhancement in killing ability of NK cells either presenting or lacking cell-surface PSA. Finally, we are using sialoside probes to assess the potential for PSA to function in cis, by masking receptors on the NK-cell surface.

In summary, we have determined that PSA expression is regulated on NK and NKT cells according to their activation state, and that this regulation may be mediated in part by the expression of novel PST transcripts. Experiments are in progress to identify a functional role for PSA expression and modulation.

(226) Novel N-glycans on Antigen-Presenting Cells Mediate Th-1 Dependent Murine Colitis

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We previously showed that carboxylated N-glycans are present on the Receptor for Advanced Glycation End Products or RAGE, a well-documented signal-transducing receptor for S100 proteins and HMGB-1 (High Mobility Group Box-1 protein or amphoterin). Since blockade of RAGE-S100A12 interaction quenches inflammatory colitis in IL-10 knock-out mice, and S100A12 binding to RAGE is dependent on RAGE glycans, we hypothesized that the glycans may have an in vivo role in the development of inflammatory bowel diseases. In support of this, we found that anti-carboxylate glycan antibody mAbGB3.1 reactivity co-localizes with S100A12 expression within mucosal and serosal inflammatory lesions and macrophage microaggregates in colon tissues from Crohn's disease patients, and also with CD80 and CD86 + activated dendritic cell phenotypes. In addition, mAbGB3.1 reactive-epitopes are constitutively expressed on macrophages and dendritic cells in intestinal and colonic lamina propria of normal mice. Most importantly, mAbGB3.1 blocks the onset of Th-1 mediated colitis. Inflammation was induced by transfer of CD4+ CD45RB^{high} T cells to Rag-1-/- immune deficient mice, and animals were monitored for weight loss and diarrhea for 6 weeks following cell transfer. Animals given the control antibody lost an average of 26% body weight and suffered from severe diarrhea. Mice treated with mAbGB3.1 showed minimal weight loss (less than 10%), were healthy, and showed no signs of diarrhea. Histopathological study revealed extensive inflammatory cell infiltration, hyperplasia, mucin depletion and crypt abscesses in control antibody treated mice, while in more than 70% of the mAbGB3.1 treated mice colonic architecture was intact with minimal or no inflammation. mAbGB3.1 specifically reduced accumulation of CD4+ T-cells in colonic lamina propria, but not in other peripheral organs such as the lung. A short-term study showed that the antibody did not inhibit either CD4+ T-cell proliferation in spleen and mesenteric lymph nodes or expression of gut-homing integrin $\alpha4\beta7$. However, colon tissues from mAbGB3.1 mice showed reduced expression of mucosal addressin molecule MAdCAM, which is largely responsible for recruiting T lymphocytes into inflamed colonic tissue, and also reduced expression of TNFα and IFNγ, products of activated macrophages and Th-1 polarized T-cells. These results indicate that mAbGB3.1 reactive glycans expressed on antigen presenting cells are involved in the development of colitis induced by T-cell transfer. Further studies are now exploring the mechanism of this inhibition. (Supported by the Broad Medical Research Program).

(227) Carboxylate Glycan-Specific Antibody mAbGB3.1 Specifically Blocks Lipopolysaccharide-Induced Proinflammatory Cytokine Production and Augments Activation-Dependent Apoptosis of Murine Macrophages

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We showed earlier that carboxylated glycans expressed on antigen presenting cells are involved in the development of colitis induced by T-cell transfer. mAbGB3.1, an antibody against carboxylated N-glycans blocks Th-1 mediated murine colitis. The antibody reduces accumulation of CD4+T cells specifically in colonic lamina propria of mice and decreases local tissue production of IFNγ and TNFα. To gain additional insight into mechanisms of antibody inhibition, we studied cytokine production by macrophages stimulated in culture. Phorbol ester-induced differentiation of HL-60 monocytic cells to macrophages dramatically increases expression of mAbGB3.1 reactive-epitopes. Receptor for Advanced Glycation End Products (RAGE) and mAbGB3.1 reactive glycans are also constitutively expressed on RAW264.7 murine macrophages. Activation of these macrophages by lipopolysaccharide (LPS) leads to the production of a variety of pro-as well as anti-inflammatory cytokines and reactive oxygen species. mAbGB3.1 specifically blocked gene expression and secretion of proinflammatory cytokines TNFα, IL-12, IL-23 and nitric oxide by LPS-stimulated macrophages, while it had minimal or no effect on the secretion of macrophage-migration inhibitory factor (MIF) or anti-inflammatory cytokine IL-10. In addition, treatment with mAbGB3.1 induced an activation-dependent apoptosis of macrophages as determined by annexin V binding, evident within fours after activation and treatment. The NF-kappaB family of transcription factors regulates the expression of a wide range of immune response genes including TNFα. Constitutive and inducible NF-κB activation also preserves macrophage survival. Our preliminary evidence suggests that mAbGB3.1 specifically blocks activation of NF-κB p65, but not other NF-κB family members p50 or Rel-B. These results suggest that mAbGB3.1 acts via blocking receptor-mediated signaling and induction of NF-κB, which subsequently blocks pro-inflammatory cytokine production and induces apoptosis. It remains to be established whether GB3.1 blocks sustained activation of NF-κB induced by RAGE ligation, or transient activation mediated by cytokine receptors or both. (Supported by the Broad Medical Research Program).

(228) Cell-type Specific Glycosylation of RAGE Influences Ligand Binding

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RAGE, the Receptor for Advanced Glycation-End Products, a major histocompatibility class III gene, is a signal tranducing receptor for a variety of structurally-unrelated ligands AGE, S100 proteins, HMGB-1 (High Mobility Group Box Protein-1 or amphoterin) and amyloid β peptide. RAGE-ligand interactions are key progression factors in the pathology of colitis, arthritis, diabetes, malignancy and Alzheimer's disease. RAGE is expressed on antigen-presenting cells and is believed to be a Pattern Recognition Receptor involved in innate immunity. RAGE is a transmembrane protein with a cytosolic tail, which has two N-glycosylation sites within its extracellular ligand binding domain. A common polymorphism of RAGE, G82S, occurs at one of the glycosylation sites and has been shown to amplify the inflammatory response. Here we show that N-glycosylation of RAGE influences ligand binding. Both N-glycosylation sites of RAGE from bovine and mouse lung are occupied; one by an Endo-H sensitive glycan chain (approximately 1.3kDa) and the other by an Endo-H resistant, PNGase F sensitive chain (approximately 2.7kDa). Bovine and mouse lung RAGE are recognized by the anti-carboxylate glycan antibody mAbGB3.1. Binding is lost upon PNGase F but not Endo-H digestion. RAGE immunoprecipitated from bovine lung by mAbGB3.1 does not express the HNK-1 epitope (3-sulfoglucuronyl β1-3 galactoside). It shows dose-dependent binding to HMGB-1 (K_d~10nM), S100A12 (K_d ~75nM) and amyloid β peptide (K_d~ 50nM). Binding to each of these ligands is inhibited by carboxylate-enriched, but not neutral glycopeptides. We could effectively deglycosylate RAGE using PNGase F without prior denaturation suggesting that the glycans are exposed and readily accessible in the native protein. Deglycosylation of RAGE reduces the binding potential (B_{max}/K_d) of S100A12 and HMGB-1. We expressed hRAGE in HEK293 and CHO cells. Extracellular domain of RAGE (soluble RAGE or sRAGE) expressed in HEK203 cells is modified by two Endo-H sensitive chains. Full length RAGE expressed in CHO cells is modified by one Endo-H sensitive glycan chain and

one Endo-H resistant, PNGase F sensitive chain. However, neither of the expressed proteins is recognized by mAbGB3.1, and both show markedly reduced binding to S100 proteins, and HMGB-1. These results show that ligand binding to RAGE is critically dependent on expression of carboxylated N-glycans. Studies are underway to establish glycan structure-function relationship of native and expressed RAGE from tumor cells, macrophages and endothelial cells. We are also investigating the involvement of other putative proteins of RAGE-ternary complexes in ligand binding. (Supported by NIH grant R01-CA92608)

(229) **Changes in Cell Surface Protein Sialylation During Maturation of Dendritic Cells and Activation of T Cells**

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Glycosylation of cell surface proteins plays a key role in immune regulation. Sialic acid is the most frequent terminal carbohydrate moiety in human glycans, playing important roles in development, homing and inflammation. Deficiencies in sialyltransferases result in immunological abnormalities in animal experiments. Sialic acid moieties serve as ligands for an expanding family of lectins. Recently, several more family members of the already established family of sialic acid-binding Ig-like lectins (Siglecs) have been cloned. Each Siglec shows distinct expression patterns in different cell types of the immune system. The presence of these receptors on antigen presenting cells as well as on effector cells suggests an important role of their ligands. Most frequently, humans conjugate sialic acids in α2,3- or in α2,6-position to the penultimate carbohydrate. We investigated the presence of these posttranslational modifications on monocyte-derived dendritic cells and on T cell subsets. We found that the sialic acid density in α2,6-binding is high on immature dendritic cells and decreases during maturation. α2,3-bound sialic acids did not change during this process. This phenomenon resulted in different affinity for influenza strains "Ukraine" and "Panama" and might have implications for the course of infection by these viruses. We stimulated peripheral blood mononuclear cells with OKT3 and IL-2 and analyzed sialylation of cell surface proteins by flow cytometry using lectins from Maackia amurensis and Sambucus nigra. We found a
- Decrease in overall sialic acid density on activated $CD8^+$T-cells.
- Differentiation of $CD4^+$T-cells into high and low sialic acid expression This finding is in contrast to previously reported reduction of sialic acid density on all T cells. This difference between cytotoxic T cells and TH cells needs to be further explored. We are separating the subpopulations and employ cytokine profiling as well as functional assays.

(230) **Ultra High-Speed Analysis of Glycosaminoglycans Using Microchip Electrophoresis**

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Electrophoresis using cellulose acetate membrane is still one of the major methods for identification of native glycosaminoglycans (GAG), although the overall procedures require at least a few hours including separation and staining procedures. Capillary electrophoresis is widely used for the analysis of wide variety of carbohydrates from glycoproteins and GAGs. A new emerging microchip technology is an attractive alternative due to its rapidness. However, carbohydrates have not been an appropriate target due to lack of chromophoric and fluorescent groups. In the present study, we propose a novel method for the analysis of GAGs using microchip electrophoresis in the presence of an intercalator reagent employed for the analysis of polynucelotides. [Methods] Apparatus: Hitachi Cosmo-i SV1100. The separation chip (30 x 100 micrometer, 5 cm length) was obtained from Hitachi. Running buffer: 0.1 M Tris-acetate buffer (pH 7.5) containing 1% polyethyleneglycol and ethidium bromide (0.001 %). Sample injection: 60 sec. Injection voltage: 300 V (injection) and 0 V (separation). Separation voltage: 130 V (injection) and 750 V (separation). [Results and discussion] Conformation of acidic polysaccharides is advantageous for complex formation with quaternary ammonium salts. In this presentation, we tried to detect intact GAGs using fluorescent conjugate formed between GAGs and a fluorescent quaternary amine during separation process, and found that almost all GAGs successfully fluoresce in the presence of ethidium bromide or propidium iodide probably due to complex formation with carbohydrate chains. Although further technical improvement is necessary to reduce the volume of sample solution for injection, we show a possibility of microchip electrophoresis to the analysis of intact GAGs. We could distinguish hyaluronic acid (pig skin, 100,000 Da) from that derived from Streptococcus zooepidemics (1,000,000 Da). The analysis is usually completed within 60 - 90 seconds. We believe that the method described here will be quite useful for high-throughput screening of biological samples and pharmaceuticals.

(231) **Glycoengineering Cancer for Its Selective Immunotherapy**

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Cancer immunotherapy is considered an ideal treatment, whereas an important problem with it is immunologic tolerance to tumor-associated antigens (TAAs). A new strategy is developed to overcome the problem, which is based upon glycoengineered modifications of cancer cells. First, an artificial analog or derivative (neoantigen) of a tumor-associated carbohydrate antigen (TACA) is applied to vaccinate cancer animals or patients. Once a specific immune response against the neoantigen is established, the animals or patients are treated with the correspondingly modified monosaccharide precursor of the TACA to initiate the exclusive expression of the neoantigen on tumor cells. Then, the pre-stimulated immune system will react to eradicate the specifically marked tumors. Immunologic studies of chemically modified GM3 antigens indicate that artificial TACAs are much more immunogenic as cancer vaccines than natural TACAs, while systemic studies of N-acyl mannosamines show that several derivatives are good precursors for the glycoengineering of cancer. Moreover, in vitro and in vivo studies of the glycoengineered immunotargeting of RMA and other leukemia tumors with α(2,8)polysialic acid as the target TACA and N-propionyl mannosamine as the bioengineering precursor proved the principle of the new strategy.

(232) **Determining the Structures on Heparan Sulfate in the Vicinity of Specific Sulfotransferase Recognition Sites by Stable Isotope Incorporation and Mass Spectrometry**

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The sulfated motifs on HS can bind to growth factors, cytokines, morphogens as well as proteases in extracellular matrix and regulate their activities, thus play critical roles in organ development, morphogenesis, angiogenesis, anti-blood coagulation, inflammation, wound healing and cancer progression, but he structures of these motifs are obscure, because of the difficulties involved in obtaining homogeneous samples of them and the difficulties involved in determining their structures. Cloning, expressing and synthesizing biopolymers have tremendously advanced our understanding of DNA and protein, but no similar methods are available for studying HS.
We have developed a strategy that permits us to quickly determine HS structures around the recognition sites of specific sulfotransferases. The strategy relies on introducing a stable isotope of sulfate onto the HS chain by the sulfotransferase. The mass-labeled HS is then digested into oligosaccharides and the oligosaccharides containing the labeled isotope are identified by mass spectrometry. The precise mass measurement tells the sizes of the oligosaccharides and number of functional groups on each oligosaccharide. The disaccharide arrangement in the oligosaccharides can be ascertained by substrate specificities of the heparan sulfate lyases, or, with the help of exoglycosidases and sulfatases. In this manner, the structure in the vicinity of the introduced isotope will be determined.
As an example, we have determined the structure of HS in the vicinity of the 3-OST-4 recognition site. 3-OST-4 is an HS sulfotransferase, which is localized in brain and sensory ganglion. Its normal physiological function is unknown, however it is able to generate a motif on HS for the entry of Herpes Simplex virus. The structure allows us to develop drugs for alleviating viral disease, inhibiting tumor growth and stimulating angiogenesis.

(233) **Genetic Basis of O-Antigen Biosynthesis of *E. coli* and Characterization of Glycosyltransferases Involved**

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Lipopolysaccharide (LPS) is an important virulence factor in Enteropathogenic Escherichia coli (EPEC). It typically consists of three components: lipid A, a hydrophobic domain; core oligosaccharide and O-antigen polysaccharide. O-antigen polysaccharide is one of the most variable cell constituents, which is

due to the genetic variation in O-antigen gene cluster. Almost all the genes responsible for O-antigen biosynthesis are cluster between galF and gnd genes in E. coli. Our studies focus on elucidation of genetic basis of O-antigen biosynthesis and characterization of glycosyltransferases involved in this biosynthetic pathway. Sequencing O-antigen gene cluster of three E. coli sereotypes (O86, O128, O127) is carried out, two of which (O86 and O128) have been completed. All the genes required for biosynthesis of O-antigen in E. coli O86 and O128 have identified by comparative sequence analysis. Study shows that four glycosyltransferase genes are involved in biosynthesis of E. coli O86 O-antigen: wbwH(encoding alpha-1,3-galactosyltransferase), wbwK(encoding alpha-1,2-fucosyltransferase), wbsJ(encoding beta-1,3-galactosyltransferase) and wbwI(encoding alpha-1,3-GalNAc transferase). Each gene is cloned and overexpressed. alpha-1,2-fucosyltransferase function of wbwK and beta-1,3-galactosyltransferase function of wbsJ have been unambiguously determined by enzymatic assay and NMR spectroscopy analysis. O-antigen of E. coli O86 and O127 are shown to have blood-group B and blood-group H activity, respectively. The elucidation of the O-antigen biosynthesis gene cluster will enhance our understanding of the relationship between human natural antibody and bacteria. More importantly, such study will further the development of efficacious carbohydrate-based vaccines against infectious bacterial diseases.

(234) Molecular and Concentration Changes in Whole Skin Glycosaminoglycans Detected by Infrared Spectroscopy

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Human skin glycosaminoglycans (GAG's) decrease with age and undergo chemical modifications, as determined utilizing highly purified molecules. In this study we report age-mediated alterations of GAG's detected in whole skin by using Fourier transform infrared (FT-IR) spectroscopy. Female, breast, mastectomy skin of individuals aged 36.33 ± 2.87 years and 77.67 ± 1.25 years was used. The FT-IR bands showing intensity differences between the two groups were quantified and assigned to known GAG and protein, functional groups. The band in the 1259-1223 cm^{-1} region that originates, among others, from the S=O stretching of sulfates (SO_4^{2-}) or sulfonates ($-SO_3^-$) decreased in intensity by 27.31% (w/w) at 77.67 years. In the 1388-1259 cm^{-1} region, there was a 33.03% (w/w) drop in band intensity in the older group. This band arises from the C-H stretch and the C-C-H vibrations of methyl moieties in monosubstituted amides as in GAG's, and from coupled vibrations of the C-C-H, O-C-H and C-O-H groups in carbohydrates. The intensity of the band characteristic of the symmetric vibrations of the carboxylate (-COO-) group (1435-1388 cm^{-1}) as in GAG's decreased by 49.90% (w/w) in the older group. There was also an 8.28% (w/w) reduction of band intensity at 1740-1646 cm^{-1} in the 77.67-year-olds. This band is produced by the C=O stretch of acetamido moieties as in GAG's, the amide I of protein, alpha helix, and the asymmetric stretching modes of carboxyl (-COOH) groups as in uronic acids. The band at 1635-1546 cm^{-1}, which is characteristic of C=C vibrational modes of Phe, Trp, Tyr and the amide I, increased in intensity by 17.00% (w/w) at 77.67 years. In addition, the band at 1511-1453 cm^{-1} produced by delta (CH2)(CH3) vibrational modes increased by 36.92% (w/w) in the older group. At 1218-1159 cm^{-1}, where bands from the C-H, the C-N, and the C-C_6H_5 vibrational modes of Tyr and Phe appear, there was a 56.54% (w/w) increment in band intensity at 77.67 years. The data show that GAG concentration decreased in the 77.67-year-old group, but protein concentration increased. These results also demonstrate the N-deacetylation of skin GAG's in the aged and confirm previous findings. The usefulness of FT-IR spectroscopy as a noninvasive technique to detect age-related, concentration and molecular changes in whole skin is also indicated by these data. The method can be used in the *in vivo* determination of molecular and concentration changes in skin aging and disorders involving GAG's.

(235) Glycosaminoglycan Raman Spectroscopy Bands, the Fingerprints of Aging

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The preponderant glycosaminoglycans (GAG's) of adult human skin, dermatan sulfate and hyaluronic acid, are known to undergo age-related alterations in concentration and chemical composition, based on the analyses of highly pure molecules. In this study we utilized Raman spectroscopy at 785 nm to determine molecular and concentration changes in whole, female, breast, mastectomy skin from two groups aged 34.00 ± 5.90 years and 74.00 ± 4.32 years. Raman spectroscopy bands that displayed different intensities in the two groups were quantified and assigned to known GAG and protein, functional groups. The intensity of the band at 584-472 cm^{-1} that has been assigned to vibrational modes of skeletal bonds in carbohydrates decreased by 22.00% (w/w) at 74.00 years. Additional bands assigned to carbohydrate vibrational modes were detected in the 676-584 cm^{-1} and 848-752 cm^{-1} regions. Their intensities in the older group dropped by 32.70% (w/w) and 18.52% (w/w), respectively. The band in the 1396-1308 cm^{-1} region includes symmetric CH_3 deformation vibrations of acetamido moieties as in GAG's, CH_2 deformation vibrations and C-O-H bending modes of carbohydrates. It decreased by 55.68 % (w/w) in the older group. There was also a 30.49% (w/w) reduction of band intensity at 1480-1396 cm^{-1} in the spectra of 74.00-year-olds. This region displays bands from the C-N stretch with contributions from C-H, deformation vibrations, and the symmetric vibrational modes of carboxylate ($-COO^-$) groups of glucuronides as in GAG's. In the older group, the intensity of the band at 1696-1600 cm^{-1} that results from C=O vibrational modes of monosubstituted amides as in GAG's and the amide I of proteins dropped by 29.90% (w/w). Another important change in the spectra of the 74.00-year-olds was the 13.36 % (w/w) reduction of band intensity at 1600-1532 cm^{-1}. This band has been assigned to N-H bendings and C-N stretching vibrations of acetamido groups with minor contributions from C-C vibrational modes, when one of the latter carbons is bonded to N as in amides. The band at 1308-1200 cm^{-1} is produced, among others, by C-N and N-H vibrational modes of protein, alpha helix and by the C-C_6H_5 stretch of Tyr and Phe. Its intensity increased by 85.63% in the spectra of the older group. The data show that GAG concentration decreased in the 74.00-year-old group, while protein concentration increased. The results also demonstrate the N-deacetylation of GAG's in aged skin and corroborate previous findings. The technique could be used at a noninvasive, light frequency for *in vivo* determination of concentration and molecular alterations of protein and GAG's. These results indicate that GAG Raman spectroscopy bands may serve as fingerprints of aging and skin disorders involving these heteropolysaccharides.

(236) Optimizing Resin Chemistry for A New, Prototype HPAE PAD CarboPac Oligosaccharide Separator Column

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We recently introduced a new monosaccharide column (CarboPac PA20; 3mm x 150mm) that gives faster, more efficient monosaccharide separations with better resolution based on a new chemistry that reduced resin particle size from 10 to 7 microns. Compared to either the CarboPac PA1 or CarboPac PA10 column, faster and more efficient separations of glycoprotein monosaccharides with better spacing were achieved across a range of isocratic NaOH concentrations at lower flow rates *. Our current efforts are directed toward developing a new oligosaccharide column with improved performance compared to the CarboPac PA100 column. The new column is being designed and evaluated with respect to giving faster, more efficient oligosaccharide separations with improved resolution based on manipulating resin chemistry and manipulating resin particle size.

* "CarboPac PA20: A New Monosaccharide Separator Column With Electrochemical Detection Using Disposable Gold Electrodes". Michael Weitzhandler, Victor Barreto, Christopher Pohl, Petr Jandik, Jun Cheng, and Nebojsa Avdalovic. J. Biochem. Biophys. Methods (2003). Submitted.

(237) Synthesis of GPI Anchor Analogs to Investigate the Biological Role of the Glycan Core

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Glycosylphosphatidylinositol (GPI) anchors are post-translational modifications that attach certain proteins to the outer leaflet of the plasma membrane. Proteins anchored through such structures play vital roles in fundamental biological processes such as signal transduction, immune response, cancer cell invasion and metastasis, and the pathobiology of trypanosomal parasites. The C-terminus of a GPI-anchored protein is linked through a phosphoethanolamine bridge to the conserved core glycan, Manα1→2Manα1→6Manα1→4GlcNα1→6-myo-inositol-1-phospholipid.

The function of the individual structural components of the core glycan has not yet been established.

To investigate the roles of the various components of the GPI anchor, we are constructing proteins bearing modified anchor structures and will study their physical and biological behaviors. Although total syntheses of GPI anchors have been reported by a few groups, these routes are not amenable to

modification and most importantly, they do not provide a method for the attachment of the anchor to the protein.
Three target GPI anchor analogs were designed to include mimics of the three domains of the native structure: the phosphoethanolamine bridge, the core glycan, and the lipid tail. These analogs contain various numbers of mannose units and will allow for the investigation of their contribution to anchor function. We will attach our GPI anchor analogs to proteins using native chemical ligation. Progess towards the synthesis and characterization of these analogs will be presented.

(238) **Specific Carbohydrate Substitution in Glycosyltransferase-Engineered Mammalian Host Cells – Production of A Recombinant Glycotherapeutic with Anti-Pig Antibody Adsorption Capacity**

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The use of recombinant proteins as therapeutics is gaining increasing interest, as indicated by the growing list of cell culture-produced products with a biological license approval. For production of recombinant glycoprotein therapeutics, mammalian host cells with the capacity to produce complex N- and O-glycans resembling the ones expressed on native human proteins have been the number one choice. CHO, BHK and murine myeloma cell lines are most frequently used, with CHO being most extensively studied in terms of e.g. glycosylation, production yield and culture conditions. However, studies of the glycosylation machinery in different cell lines have shown that others, e.g. COS and 293 cells, are superior for production of certain carbohydrate epitopes on different glycoproteins. We have developed a system, which relies on the recombinant production of mucin-type proteins, which act as scaffolds for multivalent attachment of biologically active carbohydrates. The carbohydrate substitution is determined by the host cell, which is further engineered to express specific glycosyltransferases involved in the biosynthesis of a particular carbohydrate epitope. In this case, a mucin/Ig chimera was co-expressed with the porcine α1,3galactosyltransferase (α1,3GT) in CHO-K1, COS and 293T cells in order to produce a therapeutic with the potential to adsorb natural xenoreactive antibodies recognizing terminal Galα1,3Gal. High affinity adsorbers were produced by both COS and 293T cells, while an adsorber of lower affinity was derived from CHO-K1 cells. ESI-MS/MS of O-glycans released from the mucin/Ig chimera produced in α1,3GT expressing COS cells revealed a number of structures with carbohydrate sequences consistent with terminal Gal-Gal. In contrast, no structures with terminal Gal-Gal were identified on the mucin/Ig chimera when expressed alone or in combination with the α1,3GT in CHO-K1 cells. Instead, peaks derived from mono- and disialylated core 1 structures dominated the spectra. In order to see if CHO-K1 cells could be modified to express terminal Gal-Gal epitopes on O-glycans of the mucin/Ig chimera, the α1,3GT expressing CHO-K1 cell line was stably transfected with a core 2 β 1,6 N-acetylglucosaminyltransferase cDNA. O-glycan ESI-MS/MS analysis of the resultant mucin/Ig chimera revealed a glycan composition similar to the glycans expressed on the mucin/Ig chimera produced in COS cells. Furthermore, its anti-pig antibody adsorption capacity as well as its α-Gal epitope density increased to levels comparable with the mucin/Ig chimeras produced in α1,3GT expressing COS and 293T cells. In conclusion, the density of α-Gal epitopes on the mucin/Ig chimera was dependent on the expression of O-linked glycans with core 2 structures and lactosamine extensions. Further, CHO cell-specific glycosylation could be modified to resemble the complex carbohydrate substitution of COS cells. 293 cells have been shown to produce different N-glycan structures depending on the protein expressed, e.g. sulfated as well as fucosylated GalNAcβ1,4GlcNAc. However, so far no structural analysis has been performed on the O-glycans expressed on recombinant proteins produced by 293 cells. The structural analysis of the O-glycans expressed on the mucin/Ig chimera produced in α1,3GT expressing 293T cells is currently underway.

(239) **Regeneration of Free Saccharides from Their Reductively Aminated Derivatives**

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In 1988, Kallin *et al.* [Kallin, E., Lönn, H., Norberg, T., *Glycoconjugate J.* 5 (1988) 145-150.] reported that the carbohydrate derivatives prepared by reductive amination with 4-trifluoroacetamidoaniline and cyanoborohydride can be converted to the parent saccharides by treatment with an acidic hydrogen peroxide solution with the recovery of 66 - 83%, but they have not reported the application of this method to the other derivatives. We studied the chemical regeneration of free oligosaccharides from their fluorescent derivatives prepared by reductive amination with various aromatic amines. Maltose derivatized with ethyl *p*-aminobenzoate (ABEE), 2- and 4-aminobenzonitrile (*o*-, *p*-ABN), 7-amino-4-methylcoumarin (AMC), 2-aminobenzoic acid (ABA), 2-aminobenzamide (ABAD), 2-aminopyridine (AP), and 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) were incubated with an aqueous solution of hydrogen peroxide/acetic acid at 30°C. Regeneration of maltose from ABEE, *p*-ABN, and AMC derivatives were high, giving yields of *ca.* 90%. The ABA, ABAD and *o*-ABN gave 5 to 40% of maltose, but AP and ANTS derivatives did not give maltose. However, prior treatment of AP derivative with cyanogen bromide resulted in regeneration of maltose from AP maltose in high yields. This method enabled identification of peaks of ABEE-labeled ovalbumin *N*-glycans on the free sugar basis.

(240) **Synthesis of Quinamide-Based Disaccharide Mimetics**

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Oligosaccharides constitute important recognition motifs for fundamental biological processes such as cell-cell communication, immune response, and fertilization. Whereas most protein-carbohydrate interactions are weak, a pronounced increase in binding strength can be observed frequently if oligosaccharide clusters are involved. This "glycoside cluster effect" has been the foundation of efforts for the development of carbohydrate-based vaccines and drugs. This paper reports the synthesis and characterization of Small Cluster Oligosaccharide Mimetics (SCOM). The per-*O*-acetylated derivative of L-fucose was synthesized by an improved method. Tetra-*O*-acetyl-L-fucopyranose was converted to the glycosyl cyanide by $HgBr_2$-promoted reaction with trimethylsilyl cyanide (Me_3SiCN). Reduction of the fucosyl cyanide in the presence of *t*-butyloxycarbonyl anhydride $(Boc)_2O$ yielded the corresponding Boc-protected aminomethyl-*C*-glycoside and a disaccharidic analog. Both monomer and dimer were separated by flash column chromatography and were characterized by Nuclear Magnetic Resonance (NMR) spectroscopy and Electrospray-Ionization Mass Spectrometry (ESI-MS). Removal of the acetyl groups and the Boc group towards fully deprotected aminomethyl-*C*-glycosides was investigated for both the *C*-glycoside monosaccharide and the disaccharide. The deprotected product was coupled with the previously synthesized 4,5-*O*-isopropylidene-1,3-lactone of quinic acid to yield the corresponding polyhydroxyl cluster qinamides. In separate experiments, the isopropylidenated quinic acid lactone was coupled with D-glucamine and with the previously synthesized benzyl-4,6-*O*-benzylidene-2-amino-2-deoxy-D-glucosamine to yield the corresponding quinamides. The identity of all products was confirmed by 1D-/2D-NMR spectroscopic techniques and mass spectrometry.

(241) **Synthesis and NMR-Analysis Of 2-Amino-2-Deoxy-Mannuronic Acid Derivatives**

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Aminosugars constitute abundant building blocks of naturally occurring polysaccharides or antibiotics. The most frequently found aminosugars are members of the class of 2-amino-2-D-hexoses. *N*-Acetyl-D-glucosamine for example is a major constituent of biologically important polysaccharides such as hyaluronic acid, dermatan sulfate, keratan sulfate, and is the anchor for *N*-linked glycans in many glycoproteins. *N*-Acetyl-D-galactosamine can be found in the chondroitin sulfate family and as the *O*-glycosidically linked unit in many glycosylated proteins. It has been shown that metabolic pathways of *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosamine can be exploited for cell surface engineering. Altered cell surface oligosaccharides offer thus a way for the study of cell-cell interactions. For such studies to be meaningful, it is important to have well-characterized simple carbohydrate building blocks and

synthetic strategies in hand. This paper reports the synthesis of 2-amino-2-deoxy derivatives of D-altrose, D-allose, D-glucose, and D-mannose starting from D-glucose. The protected mannosamines were subsequently converted into 2-amino-2-deoxy-mannuronic acids by oxidation. The structures of the products were confirmed by ^{1}H-, ^{13}C-, ^{1}H-^{1}H-COSY, and ^{1}H-^{13}C-COSY (HETCOR) Nuclear Magnetic Resonance (NMR) spectroscopy. Observed coupling constants were correlated with the molecule's average solution conformation by the Karplus equation.

(242) **Small Molecule Modulation of Polysialyltransferase Activity.**

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Polysialic acid (PSA) is a linear homopolymer of *f*Ñ2,8-linked N-acetylneuraminic acid found primarily on the N-linked glycans of the neural cell adhesion molecule (NCAM). In mammals PSA is expressed on embryonic tissues including the heart, muscles, kidney and brain. In the adult, PSA becomes restricted to regions of permanent neural plasticity and regenerating neural and muscle tissues. It has been speculated that polysialylation disrupts NCAM-mediated cell-cell interaction and facilitates cell migration during development and neurite outgrowth. Polysialylated NCAM is also expressed as a neoantigen in certain metastatic tumors, such as lung carcinomas, neuroblastoma, meduloblastoma, pancreatic cancer, pituitary tumors, and Wilms¡¦ tumor of the kidney. Akin to its role during development, it has been suggested that PSA on tumor cells reduces their adhesion and enhances their metastatic potential, although a direct causal relationship has been difficult to prove experimentally.

Biosynthesis of PSA in mammalian cells is carried out by two closely related Golgi resident enzymes: ST8Sia IV (PST) and ST8Sia II (STX). Both are type-II transmemberane proteins. Their localization (CTS) and catalytic (CAT) domains appear to function independently. Using chimeras comprising of the CTS and CAT domains fused seperately to FRB or FKBP, we are developing a method for manipulating the activity of PST and STX using the small molecule, rapamycin. Such an inducible system where the cell surface polysialylation can be modulated using a small molecule could be a powerful tool for studies of PSA function.

(243) **Recognition of O-glycan Clusters Synthesized Enzymatically on Mucin by Lectins**

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[Background and Aim]

Membrane-bound and secreted mucins are epithelial cell products which present clustered O-glycans toward the luminal side. Proper arrangements of glycans of mucins are thought to be critical for their interaction with carbohydrate recognition molecules, yet regulation of O-glycan attachment toward mucin polypeptides and its relationship to their affinity with endogenous or exogenous lectins are not fully understood. We previously demonstrated that there were preferential orders and maximum numbers of GalNAc incorporation into consecutive Thr residues corresponding to a portion of the MUC2 tandem repeat, which was strictly regulated by the specificity of pp-GalNAc-Ts. In the present study, a fluorescein-conjugated peptide with three consecutive Thr residues, PTTTPLK, was glycosylated by four isozymes of *N*-acetylgalactosaminyltransferases (pp-GalNAc-T1, T2, T3, or T4). Partially glycosylated peptides were purified and further incubated with a microsome fraction from human laryngeal carcinoma H.Ep.2 cells expressing β1-3-galactosyltransferase(s). Resultant glycopeptides were isolated, characterized for their structures, and tested for their interaction with plant lectins.

[Results and Discussion]

The products of cell-free GalNAc incorporation were separated by RP-HPLC and characterized by MALDI-TOF MS and peptide sequencing. All pp-GalNAc-T isozymes tested exhibited distinct specificities toward (glyco)peptides. Among them, pp-GalNAc-T2 was very unique because this enzyme transferred two GalNAc residues into Thr-3 and then Thr-2 only when a GalNAc residue was attached to Thr-4. This enzyme transferred one GalNAc residue preferentially into Thr-2 when the naked peptide was used as the substrate. The enhancing effect of the GalNAc residue at Thr-4 was completely abrogated by galactosylation of this GalNAc residue. Efficiency of galactosylation of GalNAc residues was different from each other. Galactosylation of the GalNAc residue at Thr-2 of FITC-PT*TTPLK peptide (*, *N*-acetylgalactosamine) was more efficient than that at Thr-4 of FITC-PTTT*PLK peptide. O-glycans with different arrangements on fluorescein-conjugated mucin core peptides, PTTTPLK, PT*TTPLK, PTTT*PLK, PT*TT*PLK, PTT*T*PLK, PT*T*T*PLK, PT*^{o}TTPLK, PT*^{o}TT*^{o}PLK, PTTT*^{o}PLK, PTT*^{o}T*^{o}PLK, PT*^{o}T*^{o}T*^{o}PLK, PT*^{o}TT*PLK, PTT*^{o}T*PLK, PT*T*^{o}T*PLK, and PT*^{o}T*^{o}T*PLK (^{o}, galactose), were tested for their interactions with plant lectins. *Vicia villosa* agglutinin (VVA) and peanut agglutinin (PNA), which were known to preferentially bind GalNAc and Galβ1-3GalNAc residues, respectively, were employed. Results of fluorescence polarization measurement indicated that glycopeptides with two GalNAc residues at Thr-2 and Thr-4 strongly bound VVA. The affinity was stronger than a peptide with three GalNAc residues. PNA bound more strongly to glycopeptides with two Galβ1-3GalNAc residues at Thr-2 and Thr-4 than other glycopeptides. These results suggest that VVA and PNA recognize arrangement of O-glycans on mucin peptides. We propose that this method is very useful for the analysis of interaction between glycopeptides with multiple carbohydrates and lectins in a liquid phase.

(244) **Carbohydrate Binding Specificities of New Brazilian Lectins**

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We have previously studied the carbohydrate binding properties (J Biol Chem. 2000, 275, 16119; J Biol Chem. 1998, 273, 12082.) of several new lectins, known as Diocleinae lectins, isolated from legumes grown in Brazil. Here we report the carbohydrate binding specificities of some other new lectins from Brazilian legumes. Mannose/glucose specific lectins were purified from Canavalia maritima and Canavalia boliviana, species close to ConA. Carbohydrate binding properties of these two lectins are comparable to those of ConA with certain important thermodynamic differences. The core trimannoside of N-linked oligosaccharide binds to these lectins with significantly higher Ka and enthalpy values, compared to Me-alpha-mannoside, indicating an extended site interaction with the former oligosaccharide. Like ConA, they recognized the same set of hydroxyl groups of trimannoside with some interesting variations in the thermodynamic parameters. Core pentasaccharide (Man5) and a biantennary complex carbohydrate (GnGn) also showed higher affinities for these lectins. Two other mannose/glucose specific lectins, PPL and ARL, were isolated from Parkia platycephala and Andria retusa, respectively. Unlike the lectins from the ConA group, they do not possess extended binding sites for core trimannoside and do not show enhanced affinities for Man5 and GnGn pentasaccharide. Apart from these, the seeds of Cymbosema roseum were found to contain two lectins namely, CymbLact and CymbMan that showed distinct carbohydrate specificities. CymbMan is a mannose/glucose binding lectin with an extended binding site for core trimannoside and recognizes the same hydroxyl groups of the sugar as do the other ConA group of lectins. It also interacts with Man5 and GnGn pentasaccharide. On the other hand, CymbLact is a galactose specific lectin that recognizes certain biologically important oligosaccharide structures. These lectins comprise an interesting model system to study how structurally similar lectins with similar monosaccharide binding properties demonstrate distinct specificities for larger oligosaccharides. These lectins are valuable biochemical tools as they recognize certain biologically important carbohydrate structures.

(245) **Improvement of Therapeutic Glycoproteins: *In Vitro* Remodeling and GlycoPEGylation ™**

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The glycosylation of therapeutic glycoproteins can be critical in determining bioactivity and half-life. Two strategies are presented for improving glycoprotein properties: GlycoAdvance™ and GlycoPEGylation™. GlycoAdvance™ enzymatic carbohydrate remodeling technology can produce uniform glycoforms tailored to provide optimal performance from glycoproteins from a number of expression systems including non-mammalian cells. GlycoPEGylation™ of proteins uses glycosyltransferases to introduce

PEGylated sugars which can extend circulatory half-life. Many alternative expression systems to CHO (yeast, plants, insect cells, etc.) are incapable of producing human-like glycan chains. EPO expressed in insect cells has N-glycans consisting of only the trimannosyl core, and is rapidly cleared from circulation. Using the appropriate glycosyltransferases in vitro (GlycoAdvance™), EPO with fully sialylated, branched chains can be produced. Modification of protein therapeutics with polyethylene glycol (PEG) has successfully increased half-life for drugs such as interferon alpha (PEGASYS™ and PEG-Intron™). Using glycosyltransferases to add PEGylated sugars to pre-existing glycans can extend half-life while preserving bioactivity by using attachment sites that are already modified post-translationally. GlycoPEGylation of FSH with ST3Gal3 and CMPSA-PEG 20K increased area under the curve (AUC) 4.5-fold in rats while retaining bioactivity.

(246) Cancer Antigen Synthesis Using OPopS™

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The OPopS™ technology is a proven breakthrough in computer-aided oligosaccharide synthesis. The approach is based on the reactivity difference of various sugar thioglycoside acceptor/donor derivatives (building blocks) by which complex oligosaccharide can be synthesized in a one-pot without protective group manipulation. Thereby, it enables the rapid assembly and systematic analysis of structure-function relationships for complex molecules such as oligosaccharides and sugar-containing drugs.

Because cells express unique oligosaccharide structures upon carcinogenic transformation, carbohydrate-based antigens offer the potential for a targeted immuno-therapeutic approach to the treatment of certain forms of cancer. However, these carbohydrate antigens are extremely difficult to synthesize by conventional methodology, only small quantities of these unique oligosaccharides are available for preparing potentially promising antigen constructs in order to maximize the immune response. The success of this approach relies almost entirely on the feasibility of chemical synthesis of these cancer antigens. With OPopS™ technology, Optimer Pharmaceuticals is uniquely positioned to develop these classes of compounds for use as cancer vaccines.

Globo H, a glycosyl ceramide, was isolated and identified as a carbohydrate antigen on prostate and breast cancer cells, and has been prepared by several groups via step-by-step synthesis. *Wong et al.* have described the first one-pot synthesis of Globo H hexasaccharide in one-pot manner. We have reevaluated the synthetic approach for such complex oligosaccharide and examined alternative strategy. At this meeting, we will present our study on how efficiently a complex oligosaccharide can be made by OPopS™.

(247) Trehalose Metabolism in Mycobacteria: An Unusual Pathway Involving Maltose

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Trehalose is an important disaccharide in various organisms, including mycobacteria. In Mycobacterium tuberculosis and M. smegmatis, it occurs in the cytosol and serves as an energy and carbon store, and may also be a stress protectant. It is also a major component of cell wall glycolipids in these organisms, such the toxic lipid trehalose-dimycolate.

The best known pathway for synthesis of trehalose involves transfer of glucose from UDP-glucose to glucose-6-P to form trehalose-6-P and then removal of phosphate to produce free trehalose. However, recently two other pathways for the production of trehalose have been implicated in mycobacteria based on gene homologies with pathways in other organisms. One of these pathways involves a single enzyme called trehalose synthase (TS) that interconverts maltose and trehalose.

This report describes the purification, cloning, expression and properties of TS from M. smegmatis. TS was purified from the cytosolic fraction and gave a single 67 kDa band on SDS gels, and an active enzyme of 390 kDa on a Sephracryl S-300 column, suggesting a hexamer of 6 identical subunits. Based on amino acid sequences of several peptides from the purified TS, the treS gene was identified, and cloned and expressed as active enzyme in Escherichia coli. The recombinant protein was synthesized with a (His)6 tag at the amino terminus.

The conversion of trehalose to maltose, and maltose to trehalose, by the purified TS was studied at various substrate concentrations. At 0.5 mM concentration of maltose, equilibrium was reached in about 6 hours and contained about 42% of both trehalose and maltose with about 8% glucose. At 2 mM maltose, it took 22 hours to reach the same equilibrium mixture, whereas at 2 mM trehalose, only about 30% of the trehalose was converted to about 22% maltose in 22 hours. These data indicate that maltose is the preferred substrate. Measurements of Km values also indicate that maltose is a better substrate, since the Km for maltose was about 10 mM, whereas for trehalose it was about 90 mM. TS had no activity with other trehalose anomers, i.e., á,â-trehalose or â,â-trehalose, nor with isomaltose or cellobiose. However, nigerose (á1,3-linked glucose disaccharide) was about 20% as effective as maltose as a substrate.

The enzyme had a pH optimum of about 7 and was competitively inhibited by Tris buffer. Radioactive trehalose was converted to radioactive maltose even in the presence of a 100-fold excess of unlabeled maltose, whereas radioactive maltose produced radioactive trehalose in the presence of excess unlabeled trehalose. These studies strongly suggest that TS has two distinct binding sites, one for maltose and the other for trehalose. The catalytic mechanism probably involves scission of the incoming disaccharide and transfer of a glucose to an enzyme-bound glucose, since free radioactive glucose incubated with TS and either unlabeled maltose or unlabeled trehalose results in the formation of radioactive disaccharides.

(248) Inhibition of Tumor Metastasis *in Vivo* by Disaccharide Decoy

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The cell surface carbohydrate antigen, sialyl Lewis X (sLe^X), is expressed on many carcinomas and 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 . Thus, the development of a pharmacological approach to inhibit sLe^X on tumor cells could improve patient survival. Towards this goal, we have developed a glycoside-based decoy. Synthetic disaccharide, acetylated GlcNAcβ1-3Galβ-O-naphthalenemethanol, acts as a primer of oligosaccharide synthesis, generating products related to mucin-like chains and Lewis antigens. Priming diverts the assembly of the chains from endogenous glycoproteins resulting in inhibition of sLe^X expression. Treatment of mouse LL/2 Lewis lung carcinoma cells with this disaccharide reduced their cell-surface sLe^X expression and their adhesion to purified P-selectin. Lectin staining showed the disaccharide decreased α1,3fucosylation and the inhibitory activity correlated inversely with fucosyltransferase and sialyltransferase activity based on enzyme assays. Tumor cells in a syngeneic mouse metastasis model had reduced survival, resulting in a reduction in pulmonary metastasis in disaccharide-treated mice. This is the first demonstration of sugar-based therapy using a metabolic inhibitor to reduce tumor metastasis.

(249) Engineering of Cell Surface Sialic Acids
Synthesis of Analogs of 2-N-acylamino-2-deoxy-α,β-D-mannopyranose and Studies on their Efficacy of Uptake and Toxicity

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Sialic acids, present ubiquitously on mammalian cell surfaces, play key roles in biological events, such as cell-cell and cell-matrix interactions, cell signaling, cell adhesion, and tissue growth. Numerous biological studies have shown a clear distinction in the cell surface sialylation pattern of healthy vs. diseased states. Sialic acid is bio-synthesized from N-acetylmannosamine (ManNAc) via the intracellular sialic acid pathway and expressed on the termini of the cell surface glycans (N-and O- linked). Previous studies have established that externally-supplied ManNAc (or synthetic analogues) can be taken up by a cell and be metabolically converted to sialic acid in a process becoming known as cell-surface sialic acid engineering.

Sialic acid engineering methods can be used as research tools to probe the natural functions of surface sialoglycoconjugates. In addition, efforts are underway to use these techniques to manipulate cell surface sialic acids and "repair" defects associated with human disease. Advancement of both of these research areas is currently limited by the inefficient cellular use of the free monosaccharide forms of various N-acyl derivatives of mannosamine (Acyl = acetyl, glycolyl, propanoyl, levulinoyl, azidoacetyl etc.). By contrast, the per-

acetylated analogs of these N-acylmannosamine derivatives are metabolically incorporated into cellular sialic acids with two to three orders of magnitude greater efficiency; unfortunately the acetylated compounds are toxic under certain conditions, limiting their use. Towards our goal of developing safe and efficacious ManNAc analogues for use in sialic acid engineering applications, we have synthesized a panel of analogs of (a) peracetylated N-acylmannosamine derivatives and (b) various peracyl N-acetylmannosamine derivatives. In this poster, we will present synthetic details and results from biological studies on the (a) metabolic efficiency and (b) toxicity of these compounds. Results from studies to enhance the uptake of hydrophobic N-acylmannosamine analogs, that are poorly soluble in the cell culture media, *via* micellar delivery and the consequent sialic acid production will also be presented.

(250) **Highly Sensitive Method for Mannose 6-Phosphate Quantitation Using 2-Aminobenzamide**

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A highly sensitive HPLC method has been developed to detect and quantitate fluorescently labeled mannose 6-phosphate from glycoprotein samples with limited amounts. This method is based on the mannose 6-phosphate assay currently in use (Zhou, *et al.* (2002) *Anal. Biochem.* **306**, 163-170). The method was tested on human a-galactosidase A (r-haGAL, Fabrazyme®). After acid hydrolysis of r-haGAL, the released mannose 6-phosphate was labeled with 2-aminobenzamide (2-AB) and analyzed on HPLC with fluorescent detection. Identity of the peak was confirmed to be 2-AB labeled mannose 6-phosphate. The method is sensitive for as little as 25 ng r-haGAL (approx. 1 pmol mannose 6-phosphate), which is 100-fold increase in sensitivity compared to the existing mannose 6-phosphate assay. The fluorescent intensity is proportional up to at least 2400 pmol mannose 6-phosphate ($R^2 > 0.97$). This method also proves to have good day-to-day consistency. Reproducibility was run over three assay occasions and produced a CV less than 7 %. The third assay occasion for reproducibility was also compared by the existing mannose 6-phosphate assay and the results from the two methods were very close. The method is capable of identifying mannose 6-phosphate from fluorophore-assisted carbohydrate electrophoresis (FACE) gel bands and has a good potential to quantitate the mannose 6-phosphate content in limited amount of various glycoprotein samples.

(251) **Development of Highly Active Glycomimetic Antagonists of E and P-Selectins by Targeting a Second Site with Heterobifunctional Compounds**

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Expression of E and P-selectins on the surface of vascular endothelium occurs during activation and cell division of endothelial cells. Small molecule antagonists of E and P-selectins were synthesized based on the bioactive conformation of the native carbohydrate ligand. Activities of over 500 of these rationally designed glycomimetics were confirmed by a series of independent biochemical and biological assays including the determination of IC50 values, dissociation constants (Kd), and inhibition of selectin-mediated cell adhesion under flow conditions. A new generation of potent inhibitors was developed by including the targeting of a second site for binding sulfate groups on P-selectin. Heterobifunctional bivalent glycomimetics containing haptens for both carbohydrate and sulfate-binding domains enhanced activity in vitro. One highly active antagonist for both E and P-selectins was effective in completely inhibiting inflammation in a mouse model at a dose of 1 mg/kg.

(252) **Efficient Chemoenzymatic Synthesis of poly-N-acetyllactosamine and its Fucosylated and Sialylated Derivatives**

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Poly-N-acetyllactosamine is a unique carbohydrate structure composed of N-acetyllactosamine repeats that provides the backbone structure for additional modifications, such as sialylation and/or fucosylation. These extended oligosaccharides have been shown to be involved in various biological functions by interacting as a specific ligand to selectins or galectins [1, 2, 3]. Numerous chemical approaches have been developed to prepare lactosamines by glycosylation between derivatives of galactose and N-acetylglucosamine [4, 5]. Considering the tedious multiple protection/deprotection steps involved in chemical synthesis, the amounts of products obtained in these methods hardly exceed milligram quantities. We have taken the advantage of the relaxed substrate specificity of the recombinant bacterial glycosyltransferases β4-galactosyltransferase (β4GalT) and β3-N-acetyllactosamninyltransferase (β3GlcNAcT) [6] which by concerted action generated the repeating Galβ(1-4)GlcNAc- unit. Up to tetrameric LacNAc-repeats were synthesized in multi gram amounts. The various poly-N-acetyllactosamine structures were further elongated by different recombinant fucosyl-and sialyltransferases. Based on known specificities for many of these enzymes, synthetic strategies were designed to obtain site-specific mono-, di- and tri-fucosylated polyl-N-acetylactosamine structures. These compounds are all linked to a flexible spacer that makes them suitable for practical manipulations in further biological assessments. References: [1] Ujita et al. (1999) J. Biol. Chem., 274, 16717-16726 [2] Leppaenen et al. (2002) J. Biol. Chem., 277, 39749-39759 [3] Baldus et al. (2000) Tumor Biol., 21, 258-256 [4] Aly et al. (1999) Carbohydr. Res., 316, 121-132 [5] Koeller et al. (2000) Chem. Eur. J., 6, (7), 1243-1251 [6] Blixt et al. (1999) Glycobiol. 9, 1061-1071

(253) **Human Uptake and Incorporation of an Immunogenic Non-human Dietary Sialic Acid**

Pam Tangvoranuntakul[1], Pascal Gagneux[1], Sandra Diaz[1], Muriel Bardor[1], Nissi Varki[1], Ajit Varki[1] and Elaine Muchmore[2]
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Sialic acids (Sias) are nine-carbon sugars typically found as outermost units on the mammalian cellular glycocalyx, and on secreted glycoproteins. The most common Sias are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Cellular Neu5Gc is generated by hydroxylation of the sugar nucleotide donor CMP-Neu5Ac to CMP-Neu5Gc, catalyzed by CMP-Neu5Ac hydroxylase (CMAH). Although Neu5Gc is a major Sia in most mammals (including our closest evolutionary relatives, the great apes), it is thought to be absent in healthy humans. Indeed, humans generate immune responses against intravenously administered molecules carrying Neu5Gc, e.g., the “serum sickness” reaction to equine anti-thymocyte globulin therapy. These findings are explained by a human-specific inactivating mutation in the CMAH gene that occurred ~2.5-3 million years ago. Despite no known alternate pathway for Neu5Gc synthesis in humans, antibodies have been used to claim its presence in some human cancers and in human fetal meconium. However, the specificity of the polyclonal antibodies used was not clearly defined. On the other hand, monoclonal antibodies are specific for Neu5Gc only in the context of underlying structural motifs or can cross-react with sulfated glycolipids. Although earlier studies claimed the absence of Neu5Gc from normal human tissues, we had earlier noted a small HPLC peak at the elution time of Neu5Gc in extracts from human organs. We have now explored whether Neu5Gc is actually present in normal and/or pathological human tissues, if normal humans can take up Neu5Gc from dietary sources, and if we spontaneously express antibodies against it. We have generated an antibody with high specificity and avidity for Neu5Gc via a novel method. Fetal tissues, normal adult tissues and breast carcinomas from humans showed reactivity to this antibody, primarily within secretory epithelia and blood vessels. The presence of small amounts of Neu5Gc was confirmed by mass spectrometry. Absent any known alternate pathway for its synthesis, we reasoned that these small amounts of Neu5Gc might originate from exogenous sources. Indeed, human cells fed with Neu5Gc incorporated it into endogenous glycoproteins. When normal human volunteers ingested Neu5Gc, a portion was absorbed and eliminated in urine, and small quantities were incorporated into newly synthesized glycoproteins. Neu5Gc has never been reported in plants or microbes. We found that Neu5Gc is rare in poultry and fish, common in milk products, and enriched in red meats. Furthermore, normal humans were found to have variable amounts of circulating IgA, IgM and IgG antibodies against Neu5Gc, with the highest levels comparable to those of the previously known high tittered anti-a-Gal xenoreactive antibodies. This represents a novel instance wherein humans absorb and metabolically incorporate a non-human dietary component enriched in foods of mammalian origin, even while generating xenoreactive, and potentially autoreactive antibodies against the same molecule.

(254) **Mechanism of Uptake and Incorporation of N-Glycolylneuraminic Acid into Human Cells**

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Sialic acids are nine-carbon sugars found as outermost units on the mammalian cellular glycocalyx and secreted glycans. The most common are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). The latter is widely expressed in most tissues of most mammals, with the exception of humans. Indeed, humans are unique in their inability to produce Neu5Gc, due to a mutation in the gene encoding the hydroxylase that converts CMP-Neu5Ac to CMP-Neu5Gc. Nevertheless, studies suggest that small amounts of Neu5Gc can be found in malignant human tissues. Recent data from our lab [Tangvorantukul *et al.*, 2003, PNAS, in press] showed that traces of Neu5Gc are also present in epithelial and endothelial cells of normal and fetal human tissues. However, there is no proven alternate pathway for Neu5Gc synthesis in normal or malignant human cells. We also reported that, while human carcinoma cells in culture do express moderate amounts of Neu5Gc, this is completely eliminated after several days of culture in human serum instead of fetal calf serum. Furthermore, we showed that free Neu5Gc is incorporated into cultured human carcinoma cells from an exogenous source. Thus, we hypothesize that Neu5Gc found in human tissues and tumors originates from dietary sources, particularly red meat and dairy products, which are rich in Neu5Gc. Coincidentally, red meat consumption has been associated with increased risk of certain human carcinomas, and larger amounts of Neu5Gc have been detected in these kind of cancers. It now becomes essential to better understand the uptake pathways of Neu5Gc and its potential metabolic precursor ManNGc into normal and malignant human cells, and to explore if human epithelial cells have developed specialized mechanisms for incorporation and elimination of Neu5Gc.

To address these questions, we first studied the uptake mechanism of Neu5Gc and ManNGc into human and other mammalian cells. Feeding experiments performed under various conditions with Neu5Gc allow us to conclude that the Neu5Gc uptake mechanism is not specific for human epithelial cells but also occurs in other human cells as well as in other mammalian cells. Furthermore, the uptake and incorporation of Neu5Gc is more efficient than that of ManNGc, despite the fact that the latter is smaller and less hydrophilic. We also demonstrated that the sialic acid lysosomal transporter is critically involved in the uptake of Neu5Gc, but not ManNGc. Thus we conclude that Neu5Gc enters human cells via endocytotic and/or pinocytotic processes, reaches the lysosome, is exported into the cytoplasm by the lysosomal transporter, and is then converted to CMP-Neu5Gc for eventual transfer to glycoconjugates. In contrast, ManNGc follows a different and as yet unexplained route of incorporation. These data can explain prior reports of the uptake of unnatural sialic acids into cultured cells. In ongoing studies, we have developed approaches to synthesize radioactive double-labeled Neu5Gc and ManNGc. By having a tritium label in the glycolyl group and a carbon-14 label in various ring positions, we hope to follow the fate of Neu5Gc in human and non-human cells (supported by USPHS Grant R01-GM32373, Small Glue Grant 5R24GM61894).

(255) **Novel Therapeutics from the Diversification of Heparin Structure.**

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Because the spatial orientation of arginine and lysine residues in the polyanion-binding site of heparin-binding proteins affords a surface of positive electrostatic potential it is difficult to prepare polyanions that specifically bind an individual heparin-binding protein. Heparin and heparin-like polyanions inherently bind with varied affinity to many different heparin-binding proteins, and thus protein-binding specificity is the most significant problem in developing selective heparin-like polyanions as therapeutic agents to treat a variety of disease states. In an effort to address this problem, we have been exploring new strategies to reduce or eliminate the charge dependence for saccharide-based structures to selectively bind heparin-binding proteins. In the work presented here, *N*-desulfonation of heparin has been employed to create heparin fractions having varied levels of free amine in place of *N*-sulfo groups. Substitution of the amine groups with structurally diverse moieties capable of forming protein contacts through hydrogen bond, hydrophobic and cation-p interactions using a parallel synthesis protocol provided a structurally diverse library of novel heparin derivatives. Screening of this library for binding to a panel of heparin-binding proteins using a competition-based 96-well filtration binding assay revealed unique library members possessing increased affinity and selectivity for specific heparin-binding proteins including thrombin, vascular endothelial growth factor and acidic fibroblast growth factor. Screening of the library to identify inhibitors of heparin-binding proteases revealed unique library members that inhibit the activity of thrombin, cathepsin G and leukocyte elastase. Synthesis of a second-generation library focused around a lead structure that displayed both increased affinity for thrombin and inhibited thrombin was then undertaken to investigate the structural specificity of this unique heparin-protein interaction. Evaluation of members of this focused library for binding and inhibiting thrombin revealed that unique and stringent structural requirements must be met for a non-ionic group to replace *N*-sulfo groups in heparin to achieve thrombin binding and inhibition. Further application of this library-based strategy toward the iterative replacement of charged groups on heparin and other polyanionic oligosaccharides with non-anionic groups capable of forming specific binding contacts with protein is being pursued toward the ultimate goal of identifying uncharged oligosaccharide-based structures that selectively bind specific heparin-binding proteins.

(256) **Potent Sialoside Inhibitors CD22**

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CD22, one of 11 members of the Siglec (sialic acid-binding immunoglobulin superfamily lectins) family, is a transmembrane protein exclusively expressed on B cells [1]. As a Siglec family member it binds to sialic acid containing glycoconjugates, and exhibits high specificity for those sialic acids linked to a terminal galactose residue through an α (2-6)-linkage [e.g. 2]. It is generally believed that CD22 can negatively regulate B cell signaling through association with the BCR (B cell receptor complex) following stimulation. In short, phosphorylation of the intracellular ITIM motifs of CD22 following stimulation leads to the recruitment of the phosphatase SHP-1 to the CD22/BCR complex. SHP-1 can then dephosporylate the BCR and dampen signaling [3]. Although CD22 has shown to bind to *cis* sialic acid ligands [4], it is not fully understood how the low affinity binding of CD22 to such ligands on the B-cell surface, affect its inhibitory function. It was recently demonstrated that a biphenyl derivative of the α-methyl glycoside of 9-amino-9-deoxy-Neu5Ac could increase the binding affinity to human CD22 by 200-fold relative to α-methyl-Neu5Ac, and that these analogs could partially reverse the negative regulation of B cell signaling by CD22 following activation *in vitro* [5]. We have observed that the sialoside sequence Neu5Acα(2-6)Galb (1-4)GlcNAc also has a higher binding affinity over α-methyl-Neu5Ac by about 100 fold [2]. We reasoned that the glycoside and 9-substituents of sialic acid must bind at different sites on CD22, and that the increased binding affinity they provide might be additive. Accordingly we developed an efficient method of the synthesis of 9-amino-9-deoxy-Neu5Acα (2-6)Galβ (1-4)GlcNAc [6] and prepared the corresponding biphenyl analog. Preliminary data using both ELISA-type and flow cytometric assays show that the 9-biphenyl-9-deoxy-Neu5Acα (2-6)Galβ (1-4)GlcNAc compound has 100-200 fold higher affinity compared to the unmodified trisaccharide, yielding an IC50 in ELISA assays of 400 nM. These inhibitors and analogous analogs for murine CD22 may prove useful in dissecting apart the role of the CD22-ligand interaction in regulation of B cell signaling. (Supported by NIH grant GM60938)

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(257) **Validation of Man-1-P Prodrug Therapy for Congenital Disorders of Glycosylation. A First Step.**

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Congenital Disorder of Glycosylation (CDG) Type Ia is caused by mutations in PMM2 which encodes phosphomannomutase (PMM2). This enzyme converts Mannose-6-P (Man-6-P)-->Mannose-1-P (Man-1-P), leading to synthesis of Man-containing glycoconjugates, mostly N-linked oligosaccharides. Many glycoproteins from these patients have unoccupied N-glycosylation sequons because synthesis of Dol-P-P-linked oligosaccharides is insufficient. In addition, fibroblasts from these patients synthesize truncated

lipid-linked oligosaccharides (LLO). Addition of excess Man to the culture medium of these cells increases the GDP-Man pool and normalizes LLO size. Unfortunately, application of mannose therapy to CDG-Ia patients has not been successful, despite repeated and continued efforts in a small number of patients. Another therapeutic option is needed.
Providing Man-1-P to CDG-Ia cells should bypass PMM deficiency, correct depleted GDP-Man pools, and restore normal LLO synthesis. However, Man-1-P does not cross the plasma membrane. We therefore made membrane-permeable derivatives of Man-1-P, which could be converted into Man-1-P by cytosolic esterases. Man-1-P phosphates were blocked with acetoxymethyl groups and the hydroxyls blocked by either acetyl (Compound I) or ethylcarbanate (Compound II) groups. CDG-Ia fibroblasts were incubated with increasing concentrations of either compound I or II and [2-3H]-Man to label newly synthesized LLO. Both compounds fully normalized the size of LLO. Compound II is more effective (50% correction ~ 60uM) than compound I (50% correction ~150uM). They are more efficient than correction of CDG-Ia cells by exogenous mannose when provided in a medium containing 5 mM glucose. Direct measurements showed that Compound I substantially corrects the GDP-Man pool in CDG-Ia cells, and also modestly increased pool size in normal cells.
Increasing intracellular GDP-Man pools might also normalize truncated LLOs from CDG patients with other primary defects. For instance, CDG-Ie cells have mutations in DPM1, the catalytic subunit of Dol-P-Man synthase. A homozygous point mutation (R92G) in one patient increases the Km for GDP-Man by seven-fold. We found that addition of either compound I or II at 150-300uM, fully corrects the abnormal pattern. Man-1-P derivatives may therefore have broader utility for improving impaired glycosylation resulting from other CDG defects where GDP-Man level plays a role.
Compound I did not inhibit cell growth, protein synthesis, or viability at 250uM, while Compound II showed some toxic effects above about 100uM
These results provide a starting point for further analysis of these compounds as potential therapeutics for CDG-Ia and CDG-Ie patients. Additional studies are ongoing. (Supported by RO1 DK065091)

(258) Efficient Chemoenzymatic Synthesis of Ganglioside mimics GD3, GT3, GM2, GD2, GT2 and GA2.

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Gangliosides are glycolipids that comprise a structurally diverse set of sialylated molecules. They are attached and enriched in nervous tissues and they have been found to act as receptors for growth factors, toxins and viruses and to facilitate the attachment of human melanoma and neuroblastoma cells. Specific gangliosides are also present in early stages of human neural development and affects major cellular processes including proliferation, differentiation, survival and apoptosis [1-3]. Despite the importance of these sialylated ganglioside structures, methods for their efficient preparation have been limiting. Due to recent advances in molecular biology and characterizations of various bacterial pathogens, several bacterial glycosyltransferases have been revealed and demonstrated to be very valuable tools as catalysts for glycosidic linkage formations in the preparation of synthetically defined carbohydrate structures. Recently, several glycosyltransferase genes from Campylobacter jejuni (OH4384) have been identified to be involved in producing various ganglioside-related lipo-oligosaccharides (LOS) expressed by the pathogenic bacteria [4]. Among these genes, cst-II, coding for a bifunctional α(2-3/8) sialyltransferase, have been demonstrated to catalyze transfers of Neu5Ac α(2-3) and α(2-8) to lactose and sialyllactose, respectively. Another gene, cgtA, is coding for a β(1-4)-N-acetylgalactosaminyltransferase (β4GalNAcT) is reported to transfer GalNAc β(1-4) to Neu5Acα(2-3)lactose acceptors generating the GM2 (Neu5Acα(2-3)[GalNAcβ(1-4)]Galβ(1-4)Glc-) epitope. These two glycosyltransferase genes were successfully over expressed in large scale (100 L E. coli fermentation) and used in the preparative synthesis of various ganglioside mimics. For synthetic purposes we also conducted an extensive specificity study of these enzymes using neutral and sialylated structures to further explore the synthetic utility of these enzymes. (Supported by NIH Grants GM62116 to the Consortium for Functional Glycomics, and GM60938 to JCP). References: [1] Svennerholm L., 2001, Adv. Gen., 44, 33-41 [2] Schnaar, R. L., 2000, Carbohydr. Chem. Biol., 4, 1013-1027 [3] Rampersaud, et. al. 1999, Biochem. Soc. Trans.,27, 415-422. [4] Gilbert, et. al., 2000, J. Biol. Chem., 275, 3896-3906.

(259) Probing Azido Sugar Metabolism *In Vivo* Using the Staudinger Ligation

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The ability to decorate cell surfaces with functional groups that react selectively with chemical probes has enabled novel imaging and drug delivery strategies. Our laboratory has previously demonstrated that cells in tissue culture incorporate the abiotic azido group into cell surface glycoconjugates via metabolism of unnatural carbohydrate precursors. Importantly, these cell surface azides can be selectively reacted with phosphine probes in a process termed the Staudinger ligation. This chemoselective reaction forms a covalent link between the cell surface azide and the exogenously delivered phosphine reagent. We report here the extension of this work to cell surface labeling *in vivo*. Mice were administered solutions of *N*-α-azidoacetylmannosamine (Ac_4ManNAz), and the conversion of this unnatural sugar to cell surface azido sialic acid was observed in various murine organs by Staudinger ligation analysis of isolated cell populations or tissue homogenates. Moreover, the administration of a phosphine probe to mice previously treated with Ac_4ManNAz indicated that the Staudinger ligation proceeds selectively *in vivo*. We are currently exploring the *in vivo* Staudinger ligation with radiolabeled phosphine reagents for noninvasive imaging of sialylated cells in mice.

(260) *Caenorhabditis elegans* and *Drosophila melanogaster* Lines with Defects in the Expression of UDP-GlcNAc:α-3-D-Mannoside β-1,2-*N*-Acetylglucosaminyltransferase I

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Defective protein glycosylation often results in developmental abnormalities in man and mouse. UDP-GlcNAc:α-3-D-mannoside β1,2-N-acetylglucosaminyltransferase I (GnT I) controls the synthesis of hybrid and complex N-glycans. Perturbation of GnT I is therefore an excellent way to study the function of N-glycans in metazoan development. Since the GnT I null mouse embryo dies 9.5-10.5 days after fertilization, we have initiated studies on the role of N-glycans in the development of two metazoan animal models, *Caenorhabditis elegans* and *Drosophila melanogaster*. There are three *C.elegans* GnT I genes (*gly-12, gly-13, gly-14*). We have previously reported that the *gly-13* null mutation causes partial lethality (Chen et al. Biochimie, 2003, 85: 391-401) but lethality is probably due to a second mutation close to the *gly-13* locus. We have now obtained viable worm lines with single- and double-null mutations of all three GnT I genes, including a viable *gly-13* null mutant worm line. None of these null worm lines shows any obvious phenotypic abnormality. We have also made triple-null *gly-12;gly-13;gly-14* worm lines. Homozygosity of the *gly-12;gly-13;gly-14* null worm has been proved by PCR. These worms are viable although extracts have no detectable GnT I enzyme activity. Phenotypic analysis of the triple null worm is under way. The single *D.melanogaster* GnT I gene (dGnTI) has been inactivated by RNA interference (RNAi). Viable homozygous *UAS-dGnTI-IR* transformant fly lines were obtained containing an inverted repeat (IR) of dGnTI ligated to the upstream activating sequence (UAS) promoter. These flies were crossed with heterozygous *Act5C-GAL4* flies which express yeast transcriptional factor GAL4 under the control of the cytoplasmic actin promoter. In the F1 generation of the cross, dGnTI dsRNA is expressed ubiquitously in all cells under the control of the actin promoter to induce the silencing of the dGnTI gene. We observed a 50-70% decrease in the number of viable F1 embryos and in the GnT I activity of extracts. (Supported by the Canadian Institutes of Health Research).

(261) **Characterization of Two *Drosophila* Homologs of β1,4-Galactosyltransferases**

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β1,4-galactosyltransferase (β4GalT) enzymes have been implicated in Notch protein glycosylation and function. In vertebrates, the disaccharide modification EGF-*O*-Fuc-GlcNac, generated by the enzymes *O*-FucT-1 and Fringe, can be extended by the addition of Gal in β1,4 linkage. Evidence suggests that the addition of β1,4-Gal may be important for modulation of Notch signaling by Fringe. To further investigate the role of these enzymes, we studied the function of β4GalT enzymes in *Drosophila*.

Searches of the *Drosophila* genome revealed three genes that encode proteins with homology to known β4GalT enzymes. One of these three has been identified as β4GalT7, and is involved in the synthesis of proteoglycans. We have taken a combined genetic and biochemical approach to determine the biological roles of the remaining two enzymes. We have generated mutations in both genes. Phenotypic characterization of these mutants will be presented together with biochemical analysis of the enzymes.

(262) **Acrosome Reaction-Inducing Protein-Bound Glycans in Jelly Coat of Starfish Eggs**

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Fertilization, the viaduct between generations in metazoans, defined as fusion of two sex cells, is a succession of gamete interactions, which appear to be cellularly and molecularly complex. The gamete interactions are mediated by cell surface/extracellular (egg coat) molecules, mostly protein-bound glycans. These protein-bound glycans are mostly responsible for triggering acrosome reaction (AR), a mandatory episode in gamete interactions in virtually all metazoans including mammals. The inception of AR in the starfish, *Asterias amurensis*, a good model for the study of fertilization events, was shown to be strongly associated with protein-bound glycans present in egg jelly coat. Among these molecules, an extremely large proteoglycan-like complex molecule called AR-inducing substance (ARIS) was shown to be the key component in the starfish AR. ARIS is composed of 33% (w/w) protein and the rest of sulfated sugar chains containing Fuc, Gal, Xyl, GlcNAc, and GalNAc. The sulfated sugar chains, in which one of the glycan fragments, named Fragment 1 (Fr. 1), was composed of the repeating units of [® 4]-β-D-Xyl*p*-(1→ 3)-]-]-α-D-Gal*p*-(1→ 3)-]-]-α-L-Fuc*p*-4 (SO_3^-)-(1→ 3)-]-]-α-L-Fuc*p*-4(SO_3^-)-(1→ 4)-]-]-α-L-Fuc*p*-(1→)n, (Koyota *et al.*., 1996, *J. Biol. Chem.* 272 (16), 10372-10376), were shown to be liable for the induction of AR. Recent study has shown that this sugar chain is linked to the peptide part by *O*-glycosidic linkage through a sugar chain with different structural features from Fr. 1. This inner sugar portion of ARIS was isolated as Fragment 2 (Fr. 2) from the sonicated products of pronase digest of ARIS. Fr. 2, which retains AR inducing activity to an admirable extent, having an apparent molecular size of 400kDa, and is composed of Gal, Xyl, Fuc, GalNAc and GlcNAc in a molar ratio of 5:1:5:4:2 with *O*-sulfate substitutions at Gal- 4, Gal-2, Gal-2, 3 and Gal 2, 4 (di-sulfated), Fuc-4, and GlcNAc-6. The study of Fr. 2 revealed that the major portion of the inner sugar chain of ARIS is composed of the heptasaccharide units of →)-Gal*p*-(1→ 3)-Fuc*p*-(1→ 3)-Gal*p*-(1→ 4)-GalNAc*p*-(1→ 4)-GlcNAc*p*-6(SO_3^-)-(1→ 6)-Gal*p*-4(SO_3^-)-(1→4)-GalNAc*p*-(1→ (Gunaratne *et al.*., 2003,*Glycobiology*, vol. 13, No. 8, 567-580). The study corroborated that *O*-sulfation, fucosylation and *O*-glycosylation were essential modifications of ARIS for its biological activity. Though ARIS was thought to be the only acidic glycosylated proteins in the egg jelly of the starfish for many years, very recently, we could isolate a new acidic glycosylated protein from the jelly coat of starfish egg. Our prelude study has demonstrated that this component could be somewhat responsible for the induction of acrosome reaction to a certain extent under the condition of high calcium seawater. Sugar composition analysis of this component showed that it was composed of Man, GlcNAc and GalNAc in a mole ratio of 3:5:1, which is entirely different from ARIS□fs sugar composition. Further, amino acid composition and molecular size of this component are also inconsistent with ARIS, suggesting that this is a novel glycosylated protein in the jelly coat. Further studies of this molecule are in progress.

(263) **Fucosylation and the Anti-Horseradish Peroxidase Epitope in *Drosophila melanogaster***

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Neural anti-horseradish peroxidase staining is a feature of the ecdysozoa; indeed, this staining was first described in *Drosophila* some twenty years ago and although it was surmised that this cross-reaction was due to carbohydrate, it was only in 2001 that relevant difucosylated N-glycans, carrying α1,3- and α1,6-fucose residues attached to the innermost N-acetylglucosamine residue, were detected (Fabini et al., J. Biol. Chem. 276, 28058-67). These glycans account for about 1% of the N-glycans of adult flies; recently, we have seen that these are enriched in a *Drosophila* neuronal cell line, but are absent from Schneider cells. Furthermore we have cloned four α1,3-fucosyltransferase homologues (FucTA, FucTB, FucTC and FucTD) and the single α1,6-fucosyltransferase Fuc-TVIII homologue (encoded by the *FUT8* gene). Using the *Pichia* system, we have detected core fucosyltransferase activity for both FucTA and Fuc-TVIII, which leads us to conclude that it is the combined action of these two enzymes that creates the difucosylated glycan decoration. We also transfected Schneider cells with all four α1,3-fucosyltransferase homologues and, thus far, have only detected acquisition of anti-horseradish peroxidase binding when FucTA was introduced into these cells. Subsequently we have performed RNAi on the neuronal cell line and virtually complete abolition of anti-horseradish peroxidase binding occurred when the FucTA transcripts were targeted. Thus, we believe that FucTA is a key enzyme in the biosynthesis of the anti-horseradish peroxidase epitope in *Drosophila*.

(264) **Association of *Branchless*, an FGF Ortholog of *Drosophila*, to the Cell Surface Heparan Sulfate.**

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*Branchless (bnl)*is an FGF ortholog in *Drosophila.* Histochemical analyses of its expression during development have been reported, but biochemical properties of this molecule are poorly understood. Although heparan sulfate proteoglycans play important roles in *Drosophila*development and are characterized as an important component for the FGF signaling in mammalian system, it has not been investigated whether *bnl*interacts with heparan sulfate. Here we analyzed *bnl*expression in *Drosophila*cells with the emphasis on its interaction with heparan sulfate.

The cDNA encoding *bnl*was cloned by using RT-PCR from total RNA pool of *D. melanogaster*embryo. Its expression vector was constructed and transfected into D-Mel-2 cells that were previously established from *D. melanogaster*embryo. The expressed *bnl*was successfully detected in the lysate of the transfected cells. In spite of the presence of predicted cleavable signal sequence for secretion in *bnl,* the protein was not detected in the conditioned medium even after concentration. Examination of the subcellular localization of *bnl*by subcellular fractionation, histochemical analysis and cell surface labeling revealed that the expressed *bnl*protein was specifically localized on the cell surface. The cell-surface associated *bnl*was mostly released by washing the cells with NaCl or heparin. The results suggest that *bnl*binds to heparan sulfate sugar chains and that expression and secretion of *bnl*is followed by its immediate association with the cell-surface heparan sulfate proteoglycans.

Many FGF family members in mammalian system utilize different mechanism for their secretion and/or export from the expressing cells that would ultimately regulate their availability to the cell-surface receptors for signal transduction. The results of the present study further suggest that *bnl*may function by juxtacrine mechanism. This hypothesis is currently being investigated.

(265) **May the Force and Specificity Be with Carbohydrate-Carbohydrate Interactions.**

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Direct carbohydrate-carbohydrate interactions are often considered as weak due to low association constants and therefore not able to provide required specificity for cellular recognition. In the first experimental evidence for cell-cell recognition in the animal kingdom, specificity and adhesion force was assigned to marine sponge cell surface proteoglycans. Sponge cells posses the

remarkable ability to species-specifically aggregate after mechanical dissociation to finally reconstitute a functional sponge with canals, mineral skeleton, and collagen fibrils and fibers. However, the selective species-specificity of the cell-cell interaction could not yet be pinpointed to the protein or the carbohydrate portion of the proteoglycan molecule alone and yet less to a glycan-glycan recognition process.

In this work, the strength and species-specificity of proteoglycan-mediated cell-cell recognition could be assigned to a direct carbohydrate-carbohydrate interaction. In the classical assay for specific cell-cell recognition, live sponge cells can recognize their own kind and form big homogeneous aggregates on a shaker at the right shear forces. Assays for cell-glycan and glycan-glycan recognition were designed. In a cell-glycan recognition assay, live cells, as in cell-cell interactions, demonstrated species-specific recognition and aggregation with glycan-coated beads similar in size to sponge cells. Moreover, in the glycan-glycan recognition assay, the glycan-coated bead-bead aggregation perfectly mimicked the species-specific cell-cell aggregation. A great enhancement in differentiation of specific cells, which can give rise to all cell types of a mature sponge to establish the histological system characteristic of the adult sponge, was observed when cells adhered to their own glycans coated on plastic surfaces.

At the atomic level, atomic force microscopy (AFM) measurements revealed adhesion forces between single glycan molecules in the range of 190-310 piconewtons. They compare well with the range of other biologically relevant forces measured in other recognition systems, e.g. between proteins and glycans. Quantitative measurements of adhesion forces between glycans from identical species vs. glycans from different species confirmed the species-specific character of the interaction. P values for the difference in binding forces between the two, calculated from Mann-Whitney test, were clearly below 0.01 and showed that the difference is statistically significant. Furthermore, interactions between glycans from the same species showed preferences towards a more polyvalent character of binding, which further increased the strength of the interaction and thus the specificity.

These findings significantly broaden the current views on the role of carbohydrates in cellular recognition by a novel demonstration of the species-specific character of the glycan-glycan interaction with relatively strong single binding forces in the range of several hundred piconewtons. The fact that the outermost cell surface is made up primarily of a dense layer of hydrophilic glycans strengthens the notion that upon first contact between cells such reversible and flexible glycan-glycan interactions may play a pivotal role in cell recognition processes.

(266) **Overproduction of Human GDP-fucose: Protein *O*-fucosyltransferase I and Fringe Proteins.**

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The Notch receptor is a 300 kDa single-pass trans-membrane protein involved in cell fate decisions throughout mammalian development and into adulthood. The Notch signaling pathway was first described in *Drosophila* and is well conserved throughout metazoans. Notch signaling has been implicated in numerous human diseases including certain forms of cancer, Multiple Sclerosis, Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) and Alagille Syndrome. In addition Notch plays a role in haematopoiesis. Interactions between Notch and its ligands Delta or Jagged, which are also trans-membrane proteins, results in signal transduction across the cell membrane regulating the CSL family of transcription factors. The interaction of Notch with its ligands is modulated by glycosylation of the EGF-like repeats in the Notch extracellular domain. This unusual manner of modulating signal transduction involves fucosylation of a serine or threonine residue within the consensus sequence C^2-X_{4-5}-S/T-C^3 with or without subsequent elongation to a tetrasaccharide by a Fringe (a b3GlcNAc transferase), b4GalT-1, and finally a sialyltransferase. GDP-fucose: protein *O*-fucosyltransferase I (*O*-FucT-1) is the enzyme responsible for addition of fucose to the serine or threonine residues of EGF-like repeats. *O*-FucT-1 knockouts in mice (Shi & Stanley, PNAS, 2003) and reduction by RNAi in *Drosophila* (Okajima & Irvine, Cell, 2002) exhibit Notch-like phenotypes demonstrating that *O*-FucT-1 is an essential component of the Notch signaling system. To better understand how *O*-FucT-1 and the Fringe proteins modulate Notch signaling, we are overexpressing these enzymes in a variety of systems (Chinese Hamster Ovary (CHO) cells, Sf9 cells and *Pichia pastoris*). The goal of these studies is to produce sufficient protein to perform structural analysis of these biologically significant enzymes. These studies were supported by NIH GM61126.

(267) **A Murine Model for Hereditary Multiple Exostoses (HME) Points to the Perichondrium as the Origin for Exostosis Formation while Revealing Heparan Sulfate Deficiencies**

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Hereditary Multiple Exostoses (HME) is a disease characterized by osteochondromas on the ends of long bones. HME has been linked to autosomal dominant mutations in EXT1 and EXT2, which encode subunits of the heparan sulfate (HS) copolymerase. The clinical presentation of patients can range from mild to severe disease, even in the same kindred. In order to understand how a change in heparan sulfate biosynthesis might result in exostoses, null alleles of each gene have been created in mice. (Lin et al, 2000, Dev. Biol. 224:299-311; Stickens, D., Zak, B., Wells, D., Evans, G. and Esko, J.D., unpublished). Homozygous null embryos arrest development at gastrulation, with absence of markers for mesodermal differentiation. Cells derived from these embryos fail to make heparan sulfate, demonstrating the essential role of both subunits. Cells derived from heterozygous embryos produce reduced amounts of heparan sulfate, but heterozygous embryos appear normal, develop to maturity, and reproduce. Examination of adult mice revealed that EXT1 heterozygotes rarely form exostoses (7/87), EXT2 heterozygotes form exostoses more frequently (15/96), and compound heterozygotes (EXT1+/-EXT2+/-) develop exostoses at an even higher frequency (52/149). Histological studies on exostosis sections indicate that the osteochondromas may arise from the perichondrium surrounding the growth plate. Immunohistochemistry on exostosis and wild type growth plate sections with an antibody to heparan sulfate, reveals that HS is greatly decreased in exostoses. Chondrocytes isolated from wild type and EXT1+/-EXT2+/- costal cartilage produce similar amounts of HS, however the average chain length is about one-third shorter in the mutants compared to the wild type. GlcNAc and GlcA transferase activities are reduced in EXT1+/-EXT2+/- chondrocytes compared to the wild type. These findings suggest that the level of expression of heparan sulfate may contribute to the degree of penetrance of HME.

(268) **Defective Mammary Epithelial Proliferation in Mice Containing a Tissue-Specific Deletion of a Heparan Sulfate Sulfotransferase**

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The development of mammary gland is thought to depend on the sequential action of growth factors and morphogens, including members of the EGF, TGFb, FGF and wnt families of signaling molecules. Many of these factors bind to heparan sulfate, but the functional significance of these interactions in vivo has not been elucidated. To address this problem, we have created a conditional allele of GlcNAc N-deacetylase N-sulfotransferase 1 (NDST1) in the mouse using the Cre-loxP system. Breeding animals bearing the conditional "floxed" allele of NDST1 (NDST1f/f) to mice expressing the Cre recombinase in mammary tissue (MMTV-Cre) had no effect on the viability or expected Mendelian ratio of offspring. Female offspring bred normal litters, but the pups failed to survive. However, the offspring thrived when fostered on ICR surrogate mothers, suggesting that the NDST1f/f MMTV-Cre mothers failed to lactate. Examination of the mammary glands from mature virgins demonstrated normal primary ductal proliferation and branching. However, a striking deficiency in lobuloalveolar development occurred, resulting in severe disruption of lactation. Eviction of NDST1 in the mammary epithelia lead to the production of undersulfated heparan sulfate with altered growth factor binding properties. Western blotting revealed a lack of Cyclin D1 expression that accounts for the reduced proliferation. This was accompanied by reduced anti-apoptotic signaling through the phosphorylation of AKT/PKB and an unexpected hyper-activation of the MAPK pathway (Erk1/2). These experiments demonstrate that NDST1 modified heparan sulfate is essential for Cyclin D1 expression and apoptotic suppression required for lobuloalveolar proliferation and lactation of the mouse mammary gland.

(269) **Characterization of O-glycosylation in Human ZP3 Expressed in Transgenic Mice**

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Murine sperm initiate fertilization by binding to the outer covering of the egg known as the zona pellucida (mZP). This binding is thought to require the interaction of O-glycans linked to a specific mZP glycoprotein (mZP3) with egg binding proteins coating the sperm plasma membrane. It is well established that human sperm will bind to the eggs of higher primates and humans but not murine eggs (i.e. express taxon specific binding) (1). To test the hypothesis that this specificity is protein dependent, transgenic mice were created where mZP3 was substituted with its human analogue (huZP3) (2). Eggs from these mice continue to bind murine but not human sperm. This observation implies that the O-glycans and not the protein component confer taxon specificity. However, this relationship is valid only if the O-glycans linked to mZP3 and huZP3 expressed in the murine egg acquire very similar O-glycans (3). Using ultra-high sensitivity MALDI and electrospray MS strategies, we have characterised the O-glycans in huZP3 derived from the zonae of 200 transgenic mouse ovaries and have shown that this assumption is correct. Specifically we find that the majority of the O-glycans linked to mZP3 and huZP3 are core 2 sequences terminated with sialic acid, lacNAc (Galβ1-4GlcNAc), lacdiNAc (GalNAcβ1-4GlcNAc), Galα1-3Gal and NeuAcα2-3[GalNAcβ1-4]Galβ1-4 (Sd^a antigen). Many of these terminal sequences have previously been implicated in initial and secondary murine sperm-egg binding. Core 1 O-glycans are also present and are either unmodified or are terminated with sialic acid, β-linked N-acetylhexosamine, or the Sd^a antigen. In this study, we successfully obtained ES-MS/MS sequence data from components which were of such low abundance they were not visible above background in MALDI mass profiling experiments. These sensitivity achievements were mirrored, and in some cases surpassed, in on-line nanoLC-ES-MS/MS experiments of proteolytic digests of murine and humanized ZP. The data obtained in this investigation should be very useful for future studies to determine the precise molecular basis of initial murine sperm-egg binding.

This work was supported by the Biotechnology and Biological Sciences Research Council and the Wellcome Trust (to A.D. and H.R. M.), the National Institutes of Health (HD35652) (to G.F.C) and the Elsa U. Pardee Foundation (to M.S.P.). A.D. is a BBSRC Professorial Fellow.

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(270) **Towards the Function of Protein O-glucosylation: Identification and Characterization of O-glucosyltransferase Activity and Mapping of O-glucosylation Sites in Notch1**

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O-glucosylation, a form of posttranslational modification in which glucose is directly attached to protein through *O*-linkage, has been detected in a few serum proteins (factor VII, factor IX, protein Z, and thrombospondin) and Notch receptor. The proposed consensus sequence is C^1-X-S/T-X-P-C^2, where C^1 and C^2 are the first and the second conserved cysteines of the EGF repeat. The modification typically exists in the trisaccharide form (Xyl-α1,3-Xyl-α1,3-Glc-β-*O*-Ser), but the monosaccharide form has also been found. We have identified a UDP-glucose: protein *O*-glucosyltransferase enzymatic activity responsible for adding glucose to EGF repeats (Shao *et al.*, 2002, Glycobiology **12**, 763-770). Using an *in vitro* assay with cell extract as the enzyme source, UDP- [^{3}H]glucose as the sugar donor, and recombinant factor VII EGF repeat as an acceptor, we characterized the activity showing dependence on substrate concentration, amount of cell extract, requirements for metal ions, and optimum temperature, time, and pH. We showed that the acceptor EGF repeat must be properly folded and contain the proper recognition sequence to be modified, since reduction and alkylation as well as mutagenesis of the predicted consensus site abolished *O*-glucosylation. The *O*-glucosyltransferase activity is expressed in a wide variety of cultured metazoan and mammalian cells and in rat tissues, suggesting that the modification is widespread. We decided to use NIH 3T3 cells as a source for enzyme purification because of high enzyme activity in comparison to other available cells. Purification of the activity from cell extracts using a variety of conventional and affinity approaches is ongoing. The presence of conserved consensus sites in the EGF repeats of Notch receptor and the demonstration that Notch1 is modified with *O*-glucose oligosaccharides (Moloney *et al.*, 2000, J. Biol. Chem. **275**, 9604-9611) suggest a biological role for *O*-glucosylation analogous to *O*-fucosylation of Notch1. To demonstrate which consensus sites in Notch are actually *O*-glucosylated, we are mapping the *O*-glucosylation sites in the extracellular domain of mouse Notch 1 using mass spectrometry. The above studies will help to determine the function of *O*-glucosylation in Notch and other proteins bearing the modification. This work was supported by NIH grant GM61126.

(271) **Identification and Characterization of Genes Encoding Heparan Sulfate Modifying Enzymes in Zebrafish.**

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Heparan sulfate (HS) is an unbranched chain of repetitive disaccharides which specifically binds several ligands when attached to the cell surface or secreted extracellularly. HS chains contain sulfated domains which give HS specific binding affinities for extracellular ligands and are referred to as the HS "fine structure". The fine structure is created through a five-step series of enzymatic reactions adding sulfates to yield a mature HS chain. Numerous studies have begun to characterize the biochemical nature of this pathway, including the identification of several steps having multiple isoforms with different recognition sites. Little information, however, is known about how specific HS modifications affect development. Using zebrafish (Danio rerio) as a model system, we are examining the developmental effects of disrupting the HS fine structure. We have identified and cloned twelve genes that encode the final three steps (2-OST, 6-OST and 3-OST) in the HS chain fine structure synthesis. Current analysis is focused on examining expression patterns as well as loss of function phenotypes utilizing antisense morpholino technology. Analysis of these phenotypes will be used to further understand the roles of HS in development.

(272) **Tissue-Specific Alterations in Sialylation and Sialic Acid 9-O-Acetylation in Rats and Mice. Evidence for Species-Specific Rapid Evolution of Sialylation Phenotypes**

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The expression of cell surface sialic acids is highly regulated and different cell and tissue types show marked differences in abundance, types and linkages of Sialic acids. Sialic acids are commonly attached to underlying glycan chains in α2-3 or α2-6 linkage, usually terminating chain extension. Biosynthesis of these specific linkages is dictated by specific sialyltransferases (ST)s, and the precursor or product glycan structures can be recognized by various lectin probes. Sialic acids can also be modified by changes such as 9-O-acetylation. In order to determine if there were definable intra- or inter-species differences in the expression of cell surface Sialic acids we embarked on a lectin histochemistry study. We used frozen sections of multiple organs from females and male-- adult rats , and from adults of four different mouse strains, A/JolaHsd (A/J), C57Bl6N Hsd (C57), DBA/2N Hsd (DBA), and 129/SVPasico (129). Eight lectins were used in this analysis and included SNA (Sambucus Nigra Agglutinin) which binds to sialic acids in an α2-6 linkage to Gal(NAc); Maackia amurensis Hemagglutinin (MAH) which recognizes α2-3 linked sialic acids; and , CHE-FcD which binds to 9-O-acetylated sialic acids. Relatively few differences were noted with lectin binding to the organs of the different strains of mice examined. In contrast, major and specific differences were noted, in thebinding of CHE-FcD and some of the other lectins to the various organs of the rats as compared to the same organs in mice. This study serves to underscore the need for concentrated efforts to use strain and species specific, littermate controls during the phenotypic analysis of genetically altered mice. It also shows that while there is relative conservation within one species, two related species that shared a common ancestor less than 25 million years ago can show marked differences in the expression of sialic acid types and linkages.

(273) **Characterization of the Glycans from the Aminopeptidase N1 (APN1) Receptor of the *Bacillus thuringensis* Toxin Cry1Ac.**

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The spore forming bacterium, *Bacillus thuringiensis* (Bt) produces several protoxins that are lethal to a subset of larvae from at least three taxonomic orders of insects (Cry family toxins). Bt toxins have been used for the control of pests such as the tobacco hornworm, *Manduca sexta*. The main receptor for the Cry1Ac toxin from Bt in the tobacco hornworm midgut is the aminopeptidase N 1, (APN1). Previous reports suggested that N-acetylgalactosamine is involved in the toxin-receptor binding process. In this study, we performed a characterization of the oligosaccharides involved in the interaction between APN1 and Cry1Ac. APN1 was purified from *Manduca sexta* Brush Border Membrane Vesicles (BBMV), either as a free soluble protein (115 kDa) or the GPI-Anchored (120 kDa) form. These proteins were resolved on SDS PAGE and then subjected to blotting assays with various lectins. Concanavalin A (ConA), *Dolichos biflorus* agglutinin (DBA), soybean agglutinin (SBA), *Artocarpus integrifolia* (jacalin), *Ricinus communis* (RCA), *Ulex europeaus* agglutinin (UEA) and *Maclura pomifera* lectin (MPL) bound to the 120 kDa APN, while ConA, DBA, and UEA recognized the 115 kDa. When APN was treated with PNAGase F, and/or α-GalNAcase, to Cry1Ac was drastically reduced. On the other hand, after treatment with b-N-acetylhexosaminidase or O-glycanase, Cry1Ac binding ability was not affected. PNGase F-released oligosaccharides from APN-1 were labeled with 2-aminopyridine and then analyzed by HPLC and MALDI-TOF after treatment with various exo-glycosidases. Based on these experiments a novel structure is proposed for the oligosaccharides involved in the interaction with the Bt-toxin, namely an N-linked oligosaccharide terminating in alpha-linked N-acetylgalactosamine.

(274) **Cyclin D1 as a Target of the Notch Signaling Pathway**

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The Notch gene family in mammals is composed of four members encoding ~300 kDa single-pass transmembrane receptor glycoproteins that determine cell-fate and control cell growth. Ligand binding to Notch receptors leads to proteolysis by the presenilin-containing gamma secretase complex, and translocation of the intracellular fragment of Notch to the nucleus. Notch intracellular domain (ICD) interacts with transcription factors such as CBF-1 to bring about the downstream effects of Notch signaling. Constitutively active Notch produced by chromosomal translocation, virus-induced rearrangement, or truncation, is sufficient to cause human cancer, to induce tumors in transgenic mice, and to transform human mammary epithelial cells. The mechanisms by which Notch signaling influences the cell cycle machinery to affect cellular proliferation remain unclear. However, it has been shown that induction of Notch ICD in RKE-1 rat epithelial cells activates transcription of cyclin D1 mRNA [1]. To investigate this observation further, a series of deletion constructs of the cyclin D1 promoter were tested in reporter assays with constitutively active or inducible Notch ICD, and in a co-culture assay with endogenous Notch stimulated by the Notch ligand Jagged1. A region of the cyclin D1 promoter that responds to Notch signaling was identified. It contains a CBF-1 binding site which was shown by tandem expression and mutagenesis to be functional and to be required for this element to respond to Notch signaling. To determine whether the cyclin D1 gene responds to Notch receptor activation in vivo, mouse embryos that are severely defective in Notch signaling through multiple Notch receptors are being examined. The mutant embryos lack protein O-fucosyltransferase 1 (O-FucT-1) which is responsible for the addition of O-fucose to Notch receptor EGF repeat sequences. Inactivation of the gene encoding O-FucT-1 leads to embryonic lethality at ~E9.5 and a phenotype typical of mutants lacking downstream effectors that are required for signaling through all Notch receptors [2]. Whole mount immunohistochemistry and RNA in situ hybridization are being used to determine the effects of a lack of Notch signaling on cyclin D1 mRNA and protein expression patterns during early embryonic development. [1] Ronchini C and Capobianco AJ. Mol Cell Biol. 21:5925-5934, 2001. [2] Shi, S and Stanley, P. Proc. Natl. Acad. Sci. USA 100 5234-5239, 2003. Supported by National Institutes of Health Medical Scientist Training Grant T32-GM07288 and NIH grant R01 CA 95022 to P.S.

(275) **Complex N-Glycans Are Required For Pre-Implantation Embryonic Development**

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Complex *N*-glycans are required for mammalian embryonic development since *Mgat1* gene ablation in the mouse results in embryonic lethality at ~E10 (1, 2). However, due to the presence of *Mgat1* maternal mRNA in preimplantation embryos (3, 4), a conventional knockout approach did not determine the earliest developmental stage at which complex *N*-glycans are required in development. To answer this question, we employed a *lox*P and ZP3Cre strategy to perform oocyte-specific knockout of the *Mgat1* gene. By crossing mouse strains carrying a "floxed" *Mgat1* gene, a "neo" disrupted *Mgat1* gene or a ZP3Cre transgene, respectively, we obtained female mice with the genotype $Mgat1^{F/F}$:ZP3Cre or $Mgat1^{F/Neo}$:ZP3Cre. L-PHA staining of ovary sections from these mice showed that the zona pellucida of oocytes was negative, consistent with the absence of maternal *Mgat1* mRNA. When these females were mated with $Mgat1^{+/+}$ C57Bl/6 males, a normal number of $Mgat1^{\Delta/+}$ pups were obtained, showing that complex *N*-glycans are not required for oocyte maturation or fertilization. However, when these females were mated with $Mgat1^{+/neo}$ males, about half the embryos at E3.5 showed arrested growth, most having incomplete compaction and some consisting of only 8 cells, 4 cells or even 2 cells. Meanwhile, the remaining blastocysts were normal. The abnormal blastocysts were presumably generated from $Mgat1^{neo}$ sperm, while the normal blastocysts were generated and rescued by $Mgat1^{+}$ sperm. However, when embryos from the same mating were allowed to develop to E8.5 and genotyped, we found, to our surprise, that about half the embryos were homozygous mutant, and apparently derived from mutant blastocysts. This observation shows that pre-implantation embryos lacking *Mgat1* mRNA and arrested in growth at E3.5 can implant and thereafter be rescued by factors provided by maternal uterine tissue. Our data support the hypothesis that embryos take up maternally-derived glycoproteins (3). In summary, complex *N*-glycans, while not required for oocyte maturation, fertilization or implantation, are required for timely progression through the pre-implantation stages of embryonic development.

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(276) **Oocytes Lacking Complex N-Glycans Have A Structurally Altered Zona Pellucida and Reduced Superovulatory Response but Mature Normally and are Fertilized Efficiently**

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To determine roles for complex or hybrid *N*-glycans in oocyte maturation and function we used female mice with a floxed *Mgat1* gene ($Mgat1^{F}$) carrying a Cre-recombinase transgene under the control of the zona pellucida protein 3 (ZP3) promoter. The ZP is a thick glycoprotein shell that surrounds the oocyte and is comprised of ZP1, ZP2 and ZP3 that are expressed specifically in oocytes at the beginning of oocyte growth. To confirm efficient excision of the *Mgat1* gene in oocytes, ovarian sections and ovulated oocytes were incubated with fluorescein-conjugated L-PHA to detect complex *N*-glycans. The ZP specifically bound L-PHA in oocytes from wild type or heterozygous females, but staining was absent in oocytes from mutant females. Phase contrast pictures of the zona in ovulated oocytes revealed a thin, floppy zona compared to the thick spherical layer found in wild type oocytes. Immunohistochemistry of ovarian sections detected all three ZP glycoproteins surrounding mutant oocytes but they appeared to be at lower levels in control oocytes. Western blotting was used to detect ZP3, the ZP essential for formation of the zona, in ovarian homogenates digested with or without endoglycosidase H or PNGase F. Consistent with expectations, ZP3 from oocytes lacking GlcNAc-TI migrated faster than ZP3 from control females and was susceptible to both endoglycosidase H and PNGase F digestion. By contrast, ZP3 from control

females was resistant to endoglycosidase H and susceptible only to PNGase F digestion. To determine if the altered zona pellucida of mutant oocytes was due to sequestration of ZP3 within the oocyte, *in vitro* studies were carried out using CHO cells lacking GlcNAc-TI (Lec1) transfected with *ZP3* cDNA. Western blot analysis showed no significant difference in the amount of ZP3 secreted into the medium from CHO cells compared to Lec1 cells. Despite their abnormal zona, fertilization by *Mgat1*$^{+}$ sperm rescued mutant oocytes and heterozygous embryos developed normally giving a normal litter size. However, when mutant females were superovulated, the number of oocytes collected was reduced. To determine if this decrease was due to a defect in follicle development, ovaries were collected at 48 hours after superovulation from control *Mgat1*$^{F/F}$ females and *Mgat1*$^{F/F}$ females expressing ZP3Cre. One ovary per mouse was sectioned and antral follicles counted. No difference was found between mutant and wild type females. Therefore, the decreased superovulatory response may be due to reduced ovulation or reduced migration of oocytes to the oviduct. In conclusion, oocytes lacking GlcNAc-TI have a structurally altered zona pellucida that nevertheless allows oocytes to mature and to be fertilized normally under natural mating conditions.

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(277) Altered *N*- and *O*-glycan Patterns in a *C. elegans* srf-3 Mutant

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The srf-3 mutants show altered lectin binding affinity to wheat germ agglutinin, traction abnormalities, increased fragility and resistance to *Microbacterium nematophilum* infection (1). Here we present a comparative study of the *N*-and *O*-linked glycans of *C. elegans* srf-3 and its parent strain N2 Bristol. Neutral hexose released as *N*-glycan by PNGases F and -A was 29% and 76% of total glycoprotein releasable neutral hexose from N2 and srf-3 respectively indicating a dramatic change in *N*- and *O*-linked glycan ratio. The composition of the srf-3 permethylated *N*-glycans determined by MALDI-TOF MS show an absence or loss of glycans consistent with those previously reported to contain sub-terminally and terminally linked Gal and Fuc respectively (2,3). The GC/MS monosaccharide analysis of released *N*-glycans show a decrease in Fuc in srf-3. A series of major *O*-glycans of *C. elegans* have been reported (4). A subset are mucin-like glycans containing a GalNAc core substituted with branch forming Gal, GlcU, Fuc, MeFuc and Glc The srf-3 mutant's glycoprotein extract contained a nearly three fold reduction in releasable *O*-glycan compared to its parent strain. Other than a decrease in abundance, the permethylated neutral *O*-glycans of srf-3 were similar to those of N2 Bristol up to Hex4HexNAc1 by MALDI-TOF MS analysis. However, GC/MS analysis revealed decreased Gal in srf-3. The mutant also shows a loss of *O*-linked Hex5HexNAc1 and the charged compound(s) HexU1Hex4HexNAc1. These results suggest that srf-3 mutants contain a genetic lesion affecting both *N*- and *O*-glycan synthesis. The alterations in the distribution and composition of the glycans suggest truncation affects due to loss of Gal and, possibly, other monosaccharides. The loss, therefore, occurs upstream of the division between these pathways. Possible explanations for the observed phenotype are discussed. 1. Hodgkin, J., Kuwabara, P. E., and Corneliussen, B. (2000) Curr Biol 10, 1615-1618 2. Haslam, S. M., and Dell, A. (2003) Biochimie 85, 25-32 3. Haslam, S. M., Gems, D., Morris, H. R., and Dell, A. (2002) Biochem Soc Symp, 117-134 4. Guerardel, Y., Balanzino, L., Maes, E., Leroy, Y., Coddeville, B., Oriol, R., and Strecker, G. (2001) Biochem J 357, 167-182

(278) α-Mannosidase Iix Is Responsible for An Alternate *N*-glycan Processing Passway to α-Mannosidase II *In Vivo*

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Previous study of α-mannosidase-II (MII) gene knock-out mouse revealed the existence of an alternative pathway for MII in the *N*-glycan processing (1). α-mannosidase-IIx (MX) is one of candidate enzymes for the alternative pathway. MX protein is highly homologous to MII, and exhibited an α-mannosidase activity *in vitro*. MX-deficient mice showed male infertility due to the reduction of a GlcNAc-terminated *N*-linked carbohydrate structure, which is presented on male germ cells and is important for cell-cell interaction with supporting Sertoli cells. This result also indicated that MX is involved in *N*-glycan biosynthesis *in vivo* in the testis (2). To elucidate the role of MX in *N*-glycan biosynthesis, 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 and liver tissues of DKO, suggesting that inactivation of both MII and MX enzymes leads significant damages to these tissues, which were not found in either MII-deficient or MX-deficient mutants. We also analyzed *N*-glycan structures from DKO by lectin staining, 2-dimensional HPLC and mass spectrometry, and found no complex type *N*-glycans in DKO. These results clearly indicate that MX is the enzyme that is responsible for the alternative pathway.

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(279) Characterization of POMT2, A Novel Member of the PMT Protein O-Mannosyltransferase Family Specifically Localized to the Acrosome of Mammalian Spermatides

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Within the last few years it has emerged that O-mannosyl glycans are not restricted to yeasts and fungi, but are also present in higher eucaryotes including humans. There they play a substantial role in the onset of muscular dystrophy and neuronal migration disorders like muscle-eye-brain disease and Walker-Warburg syndrome. Protein O-mannosyltransferase genes (*PMTs*) are evolutionarily conserved from yeast to man, however, very little is known about these enzymes in higher eucaryotes. We have cloned the first PMT2 subfamily members from human (*hPOMT2*), mouse (*mPomt2*) and *Drosophila* (*DmPOMT2*). A detailed characterization of the mammalian *POMT2*, with emphasis on mouse *Pomt2*, shows that mammalian *POMT2* is predominantly expressed in testis tissue. Due to differential transcription initiation of the *mPomt2* gene two distinct mRNA species are formed which vary in length. The shorter transcript is present in all somatic tissues examined. Expression of the corresponding *hPOMT2* cDNA in mammalian cells identified POMT2 as an integral membrane protein of 83 kDa localized to the endoplasmic reticulum. The longer *mPomt2* transcript is restricted to testis and encodes a deduced testis-specific *m*POMT2 protein isoform. *In situ* hybridization and immunolocalization demonstrate that in testis tissue POMT2 localizes to maturing spermatids and is abundant within the acrosome, a sperm-specific organelle that is essential for fertilization. Our data suggest a novel and specific role for POMT2 protein O-mannosyltransferase in the maturation and/or function of sperm in mammals.

(280) Abundant and Unusual *N*-Linked Glycans from the Eukaryote, *C. elegans*

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Model organisms play a central role in making genes and their products amenable to understanding biological function. Usually, a candidate gene is eliminated (KOs or RNA*i*) and the consequence evaluated by a loss of function. *Caenorhabditis elegans* offers several advantages to study this interrelationship with knockout screens and large-scale RNA*i* approaches frequently coupled with the simplicity of microtiterplate assays. It has been our goal to support GnT knockout studies by defining glycome modulation; a chemo-phenotype. Efforts to establish a glycome composition for the worm have been extensive and was presented earlier, (H. Geiser, et al, Glycobiology 12:650:03).

As a consequence of that effort, we now report the details of a unique and new set of *N*-glycans structures not reported to be found in eukaryotic cells. The glycans, released by hydrazinolysis, showed endogenous methylation, antenna and core fucosylation with the latter structures capped with galactose. These highly core-substituted glycans were notably resistant to either endoglycosidase F or A release. Such an array of multi-positioned substituents within a MS profile produced numerous isobars corresponding to the compositions: $GlcNAc_2Hex_{4\text{-}8}Fuc_{2\text{-}4}$. However, these structures were resolvable by ion trap mass spectrometry.

The reducing and non-reducing termini were observed in MS^2 as a consequence of facile glycosidic cleavage between GlcNAcs of the core. The presence of multiple isomers was confirmed by comparing MS^2 spectra from permethylated glycans with spectra from the same set of glycans that were reduced to the alditols prior to methylation. Using this approach reducing end fragments were clearly revealed by a +16 Da mass shift that confirmed the

core substitution patterns. The complementary reducing and non-reducing end fragments from isomeric glycans were subsequently followed in MS^3 and MS^4 to reveal further structural details. One of the new reducing end moieties was found to have a ß1-4 galactose linkage on the 6-fucose of the protein linked GlcNAc as confirmed by a combination ß-galactosidase treatment and MS^n.
The novel glycans were obtained from purified protein by hydrazinolysis (100ºC for 6 hours) releasing a glycan pool composed of fucose, galactose, mannose and *N*-acetyl glucosamine. The presence of GlcNAc and absence of GalNAc suggested an absence of *O*-linked structures. MS^n revealed branching and linkage details of the *N*-glycans, including isomeric structures having extensive fucosylation with these unusual reducing termini. Data indicating these heterogeneous structures have also been analyzed using new bio-informatics tools developed in our lab, (Hailong Zhang , adjacent poster). In support of these efforts we are also building a spectral library to expedite identification of specific disaccharide linkages generated during MS^n and extend the capability of MS^n for complete structure determination.
Experiments were carried out using a Thermo-Finnigan LCQ and a Kratos MALDI-IT mass spectrometers. Supported by BRIN-NCRR(VR) and NIGMS(VR).

(281) The Pleiotropic *srf* Mutants in *C. elegans*: A Link between Expression of Surface Glycoconjugates and Intercellular Signaling During Development

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The body of *C.elegans* is surrounded by a complex cuticle, which acts as a barrier to the environment and is also relevant in locomotion and maintenance of body morphology. Collagen-like proteins constitute the major components of the cuticle; mutations affecting collagen-like proteins sometimes produce striking body shape changes, such as Dpy or Rol. In contrast, little is known about other surface associated proteins, which have been identified by lectin or antibody binding to the surface of live nematodes and by differential solubility after radiolabeling in non-penetrating conditions. *srf-4, srf-8* and *srf-9* (surface) mutants were isolated based on their ectopic surface binding to the lectin wheat germ agglutinin (WGA). In contrast to *srf-2, srf-3, srf-5* and *srf-6* mutants, which have no gross morphological defects, *srf-4, srf-8* and *srf-9* mutants have multiple defects, including uncoordinated movement, protruding vulva, abnormal egg laying, and abnormal copulatory bursae and gonad morphology, and therefore have been categorized as pleiotropic mutants (1). Each of these pleiotropic mutations enhances the defects associated with elevated activity of LIN-12, a member of the LIN-12/NOTCH family of receptor proteins that mediate cell-cell interactions to specify cell fate during development (1, 2). A broad underlying defect in the pleiotropic mutants, which would interfere with the proper expression of one or more cuticle component, may also affect other processes required for normal development. Glycosylation, protein targeting, sorting, secretion or assembly of extracellular matrices are among the potentially affected processes.
Genetic mapping located *srf-4, srf-8* and *srf-9* to specific intervals of LGV (1). In order to identify these loci, we used a candidate gene approach: the physical intervals that contain each of the pleiotropic *srf* genes were searched for ORFs encoding products with homology to known components of glycosylation and secretion pathways in other organisms. We determined that the hallmark phenotypes of the pleiotropic *srf* mutants can be induced by interference with the expression of members of a protein family involved in the early steps of the secretory pathway. In addition, genetic lesions consistent with complete loss of function of members of this protein family were detected in the mutants. Genetic interaction experiments indicate that the ability to restore and maintain ER homeostasis is critical when function of this protein family is compromised.
(1)Link C. et al (1992) Genetics 131: 867-881
(2)Sundaram and Greenwald (1993) Genetics 135: 755-763
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(282) Biochemical Characterization of *Drosophila* Sialyltransferase

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Sialylation plays a crucial role in a variety of molecular interactions. A number of pathological conditions have been linked to altered sialic acid metabolism, however the exact role of this carbohydrate in the etiology and manifestation of different diseases is still unknown. Because of their abundant presence in deuterostomes it is assumed that sialic acids were essential during the evolution of this lineage of animals, however, the recent report of a functional sialyltransferase in *Drosophila melanogaster* (D. SiaT) indicates that sialylation must have appeared already during the evolution of protostomes. Sequence comparison to other sialyltransferases revealed highest homology to the recently cloned human ST6Gal II sialyltransferase. Homologous gene has also been identified in the mosquito genome; it encodes protein with 47% sequence identity to *Drosophila* sialyltransferase. Sequence comparison to other cloned sialyltransferases suggests the possibility that insect sialyltransferases and vertebrate ST6Gal sialyltransferases have a common ancestral gene. Thus, ST6Gal family probably represents the most ancient type of animal sialyltransferases that gave rise to other vertebrate sialyltransferase families during evolution.
We characterized the acceptor specificity of D. SiaT.Ê It is unique among the sialyltransferases characterized so far. It prefers LacdiNAc containing oligosaccharides and glycoproteins, although it can also sialylate both LacNAc (type II) and type I termini. This preference for LacdiNAc termini may reflect the evolution of animal glycomes, since LacdiNAc is more abundant in protostomes than LacNAc, whereas LacNAc (the preferred substrate of vertebrate ST6Gal sialyltransferases) is more commonly found in deuterostomes. Kinetic data also indicated a preference for oligosaccharide, as opposed to glycoprotein acceptors. Glycolipids did not serve as substrates for the enzyme, including *Drosophila* glycolipids known to carry LacdiNAc termini.
Thus, D.SiaT represents the first characterized sialyltransferase in protostome lineage of animals. Given the advanced genetic repertoire of the *Drosophila* system, the evolutionary and biochemical aspects of sialylation can now be addressed from a new vantage point.
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(283) Proteoglycan , Cell Viability and Metabolic Activity in Meniscal Tissue after Preservation at Different Concentration of Formalin in PBS

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Meniscal cartilage is a unique structure in its ability to tolerate millions of steps per year and yet still remain structurally intact and protective of the joint surfaces .The meniscus also provides stability to the knee joint , lubricates it by boundary lubrication and fluid film lubrication , function as a spacer , increases joint congruency ,improves articular cartilage nutrition and facilitates the rotation of opposing articular surfaces of the joint . Menisci composed of collagen (98% type I), extracellular matrix includes proteoglycans,glycoproteins, and elastin; chondrocitin sulfate is dominate glycoaminoglycoside in aggregating proteoglycan macromolecule.Proteoglycans are the important component of the extra-cellular matrix .Proteoglycans are a class of glycosylated proteins which have covalently linked sulfated glycosaminoglycans, (i.e., chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, keratan sulfate). Proteoglycans (10-15% wet weight) has a half life of three months and regulate matrix hydration by providing a porous structure to trap and hold water. The study was designed to analyze the rabbit meniscal cell viability and metabolic activity by using different methods after preserving the menisci (n=24) , at different concentration of formalin (0.25 % , 1 % and 5 %) in PBS .The different techniques utilized in the study were to study proteoglycans by S35 -uptake assay , metabolic activity measurement in terms of LDH , Nitric oxide (NO) was measured in form of RNI (reactive nitrogen intermediates) and Citrulline and cell viability in terms of apoptosis using TUNEL(TdT-mediated dUTP nick end labeling) and by trypan blue assay .Our results shows that the menisci preserved in 1 & 5 % formalin in PBS , however showed statistical decrease in the radioisotope uptake by their fibrochondrocytes , implying loss of cell viability in them .However , there was no statistical significant evidence of cell viability survival in the menisci preserved at 1 and 5 % formalin in PBS as compared to controls .Besides that the trypan blue assay also proved that cell viability reduced to a great extent in case of 1 and 5 % formalin in PBS as compared to 0.25% and controls .The rate of cell viability was very close in control as well as in 0.25% group .The NO level was also studied and it was observed that the level of RNI and Citrulline was high in case of 1 and 5 % formalin group as compared to 0.25% and control groups . Production of NO in may be an important feature of human and rabbit menisci to study the degradation as the previous study also proved that increased production of NO is associated with matrix degradation and chondrocyte apoptosis. TUNEL assay shows that the number of apoptotic cells was very high in case of 1 and

5% formalin in PBS as compared to controls and 0.25% group. Our study suggest that the best concentration for preservation of rabbit menisci is 0.25% formalin in PBS , as this is the concentration at which cells have their maximum cell and metabolic activity with minimum level of free radical production and programmed death .

(284) **Neuronal Requirement for Hybrid N-Glycan Branch Structures**

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The Mgat1 and Mgat2 genes encode glycosyltransferases essential during the biosynthesis of hybrid and complex N-glycan branching patterns. Mgat1 deficiency disables both hybrid and complex N-glycan formation, while Mgat2 deficiency disables only complex N-glycan formation. Inheritance of the Mgat1 null genotype results in embryonic lethality with defects in neural tube development, vascularization, and situs inversus of the heart. Inherited deficiency of Mgat2 function is compatible with gestation but results in a severe disease termed Congenital Defect of Glycosylation Type IIa. Modulatory functions of N-glycans in the nervous system have been suggested from these and other findings. We have investigated structure-function relationships among hybrid- and complex-type N-glycans among neuronal cells in vivo by using Cre-loxP conditional mutagenesis. Synapsin-1 promoter was used to express transgenic Cre recombinase, resulting in neuronal-specific deletion of the loxP-flanked Mgat1 or Mgat2 genes. All embryos developed to term, however the specific loss of neuronal Mgat1 led to runting with muscular atrophy, paralysis, and death by 8 weeks of post-natal age. Astrogliosis and neuronal apoptosis were noted along with reduced expression of key neuronal and synaptic glycoproteins. Neuronal-specific deletion of the Mgat2 gene did not effect neuronal cell viability, and such mice were indistinguishable from normal littermates. Our findings reveal essential roles of hybrid N-glycan branching patterns in neuronal function and post-natal viability, while complex N-glycan branch patterns are dispensable.

(285) **Presence of a Novel GM2 Derivative, Taurine-conjugated GM2, in Tay-Sachs Brain**

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Tay-Sachs disease (TSD) is a classical inborn lysosomal glycosphingolipid (GSL) storage disease characterized by massive cerebral accumulation of ganglioside GM2 due to the deficiency of either beta-hexosaminidase A or GM2-activator protein. Although the genetic and biochemical bases for TSD have been well established, the mechanism that leads to the clinical and neuropathological manifestations in TSD is still not fully understood. Since GM2 is a normal cell membrane component, the severe neurodysfunction found in TSD cannot be solely attributed to the cerebral accumulation of GM2. Based on the premise that GSLs specifically found in TS brain are associated with the pathogenesis of TSD, we carried out the studies of GSLs in brain samples from patients with TSD. This report describes the detection and structural elucidation of a novel GM2 derivative in TS brain samples. Using NMR spectroscopy, mass spectrometry, and chemical synthesis, the structure of this unusual GM2 derivative isolated from a 75-g variant B TS brain sample was established to be a taurine-conjugated GM2 (tauro-GM2) in which the carboxy group of Neu5Ac is amidated by taurine. We have also raised a rabbit anti tauro-GM2 serum and immunologically detected the presence of tauro-GM2 in the additional small brain samples (1 – 2 g) from one variant B and two variant O TSD patients. Under the same conditions, we did not detect the presence of tauro-GM2 in three normal human brain samples. Taurine, one of the most abundant free amino acids found in the human central nervous system (CNS), has been shown to play a wide variety of biological functions, including bile acid and xenobiotic conjugation, osmoregulation, and calcium modulation. Taurine-conjugation is a well-known mechanism in biological systems to facilitate the clearance of xenobiotics from the body by increasing their polarity and aqueous solubility. Neural tissues may regard the massively elevated GM2 as a quasi-xenobiotic and employ taurine conjugation as a vehicle for its removal. The presence of tauro-GM2 in TS brains, but not in normal brains, indicates the possible association of this unusual GM2 derivative with the pathogenesis of TSD. It should be pointed out that the taurine-conjugated Neu5Ac is a novel sialic acid derivative that has not been previously revealed.

(286) **The Signaling Activity of Soluble ICAM-1 in Mouse Astrocytes depends on the Presence of Sialylated Complex-type N-glycans**

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Intercellular adhesion molecule-1 (ICAM-1) is expressed on cell surfaces and also released as a soluble glycoprotein (sICAM-1). ICAM-1 on the surface of endothelial cells mediates leukocyte adhesion by binding to leukocyte function associated antigen-1 (LFA-1) and macrophage antigen-1 (Mac-1). The biological functions of sICAM-1 are less well understood. We recently reported that sICAM-1 is strongly increased in the cerebrospinal fluid of patients with severe brain trauma and that recombinant mouse sICAM-1 induces the production of macrophage inflammatory protein-2 (MIP-2) in mouse astrocytes. The present study was aimed at defining whether glycosylation of sICAM-1 contributes to its signaling functions. Unexpectedly, sICAM-1 expressed in CHO cells was a much more potent inducer of MIP-2 production than sICAM-1 expressed in 293 cells. This finding prompted us to specifically modify the glycosylation of sICAM-1 from CHO cells using the Lec 2, Lec 8, and Lec 1 mutants of CHO cells, as well as the mannosidase I inhibitor kifunensine. Signaling activity of sICAM-1 lacking sialic acid was reduced three-fold compared to fully glycosylated sICAM-1 from wild-type CHO cells. While activity of sICAM-1 lacking both sialic acid and galactose was reduced twelve-fold, activity of sICAM-1 carrying only high-mannose type N-glycans was reduced between twelve and twenty-six fold. By contrast, the sICAM-1 glycoforms carrying truncated glycans and the fully glycosylated sICAM-1 bound equivalently to LFA-1. Taken together, these data show that glycosylation of sICAM-1, and in particular sialylation and galactosylation, enhance the ability of sICAM-1 to induce MIP-2 in mouse astrocytes. Since glycosylation of sICAM-1 does not affect its ability to bind LFA-1, glycosylation may be a means to specifically regulate the signaling function of sICAM-1 in vivo.

(287) **Establishing A Correlation Between Mutant Forms of *GNE* Found in HIBM and the Expression of Neurotrophic Factors**

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Hereditary inclusion body myopathy (HIBM) is a unique group of neuromuscular disorders characterized by adult onset and typical muscle pathology. Recent genetic studies have linked mutations in the bifunctional enzyme UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (*GNE*) with the occurrence of HIBM in Middle Eastern and other patients. So far, a total of 20 *GNE* mutations, localized to both the epimerase domain and kinase domains of *GNE*, have been identified in HIBM patients. Although *GNE* mutations found in HIBM modestly reduce metabolic flux into the sialic acid biosynthetic pathway, it is unlikely that abnormalities in sialic acid metabolism account for cellular effects, such as inclusion body formation, associated with this disease. In order to reveal the mechanism of HIBM, we transfected wild-type and mutant forms of *GNE* into human neuroblastoma cells and quantitatively compared the gene expression level of neurotrophic factors in these cells by real-time PCR. The expression level of several neurotrophic factors was significantly down-regulated by expression of *GNE* with epimerase domain mutations. For example, brain-derived neurotrophic factor (BDNF) expression was down-regulated significantly by the mutations at nucleotide 455 (G134V) and 968 (R306Q). Neurotrophic factors are growth factors or cytokines that are inducible polypeptides and permit intercellular communication, mediate neuronal cell proliferation, differentiation, maintenance, repair, and regeneration. Disruption of their normal functioning through the action of mutant forms of *GNE* offers an important insight into the cellular basis of HIBM.

(288) **A Role for Utrophin in the Carbohydrate-Mediated Expression of Synaptic Extracellular Matrix Proteins in Skeletal Muscle.**

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A number of important events in the development of the neuromuscular junction require the expression of synaptic extracellular matrix proteins, including synaptic forms of laminin. We recently demonstrated that ectopic expression of a synaptic carbohydrate, the CT carbohydrate antigen (GalNAcß1,4[NeuAc(Gc)α2,3]Galß1,(3GalNAc or 4 GlcNAc)), can cause the extrasynaptic expression of several such synaptic laminin chains as well as utrophin, a normally synaptic paralog of dystrophin (Xia et al. (2002) *Dev. Biol.* **252**, 58-73). This was done by making transgenic mice that overexpress the cytotoxic T cell (CT) GalNAc transferase (or Galgt2), a normally synaptic glycosyltransferase, specifically in skeletal muscles. In addition to altered expression of laminins and utrophin, these mice have altered muscle growth, neuromuscular structure, and neuromuscular topography. To determine if the ectopic expression of utrophin contributed to these phenotypes, we created utrophin-deficient Galgt2 transgenic mice (Galgt2utrn-/-). Muscles from Galgt2utrn-/- mice still showed reduced myofiber diameters, aberrant neuromuscular structure, and altered synaptic topography, but the topographic distribution of neuromuscular synapses was restricted in utrn-/- muscles (relative to utrn+/-) and the number of muscle spindles was increased in Galgt2 transgenic muscles (relative to non-transgenic muscles). α dystroglycan was glycosylated with the CT antigen in Galgt2utrn-/- muscles, however, the Galgt2-dependent overexpression of laminin α4 and α5 was reduced by over half in the absence of utrophin. Galgt2 transgene expression also caused the increased expression of agrin and a form of neuregulin, and their increased expression was completely utrophin-dependent. The expression of the ErbB2 and ErbB4 tyrosine kinases was also affected; ErbB4 expression was reduced in Galgt2 transgenic muscles, and ErbB2 expression was reduced in utrn-/- muscles. These data demonstrate that utrophin plays a role in the carbohydrate-mediated expression of synaptic extracellular matrix proteins, and suggest that utrophin is part of a novel synaptic glycoprotein complex that contains the CT antigen.

(289) **Fibrillogenesis of Amyloid Deposits and Neurofibrillary Tangles in the Aging and Diseased Brain**

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Two hallmarks of Alzheimer's disease (AD), amyloid plaques and neurofibrillary tangles (NFT) are labeled by calcofluor, a stain used to detect chitin in the cell wall of fungi. Neuritic plaques of late onset AD are commonly stained by two other histological dyes, congo red and thioflavin, both of which, as we showed, stain chitin. But calcofluor is more sensitive than the other two dyes because it also stains cotton wool plaques in early onset AD as well as single fibrils that densely permeate the early and late onset AD brain, in addition to the NFTs. The components that have been largely studied, so far, in amyloid plaques and NFTs are amyloid β peptides and hyperphosphorylated tau, respectively. NFTs are initiated intracellularly in neurons. While amyloid β peptides are also generated intracellularly from proteolytic cleavage of the amyloid precursor protein, the question still remains where amyloid plaques are initiated, intra and/or extracellularly. Of relevance might be our preliminary finding that in the aged control brain calcofluor-stained fibrils enmesh lipofuscin granules that are lysosomal in origin. Extracellular chitin fibrils could be generated by hhas1, a hyaluronan synthase with strong homology to chitin synthases, which was shown to synthesize chito-oligosaccharides in vitro. On the other hand, the intracellular fibrils may be the result of lysosomal dysfunction, lysosomal hypertrophy or leakiness of lysosomal membranes, which is thought to precede AD pathology. Lysosomal or cytoplasmic glycosidases may cleave sugar residues that modify soluble chitin-like polysaccharides (Huang et al.) thereby rendering them insoluble. The advent of intra- and extracellular chitin fibrils may serve as a scaffold against which other molecules, among them amyloid β peptides and hyperphosphorylated tau, precipitate in dying neurons.

(290) **Molecular Dissection of A Polysialyltransferase, ST8Sia IV: Domains Distinctively Required for NCAM Recognition and Polysialylation**

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Polysialylated NCAM is thought to play critical roles in neural development. Two polysialyltransferases (ST8Sia II and ST8Sia IV), which belong to the sialyltransferase gene family, synthesize polymer of α2,8-linked sialic acid (polysialic acid) to the neural cell adhesion molecule (NCAM). While ST8Sia III, moderately homologous to ST8Sia II and ST8Sia IV, adds oligosialic acid to itself and other molecules, but not to NCAM. On the other hand, α2,3- and α2,6-sialyltransferases can add only one sialic acid to the acceptor molecules. Here, we analyzed various recombinant enzymes to find domains required for NCAM recognition and polysialylation. We first determined a catalytic domain of ST8Sia IV using deletion mutants. To identify domains responsible for NCAM polysialylation, different segments of the ST8Sia IV catalytic domain were replaced with corresponding segments of ST8Sia II and ST8Sia III. We found that larger polysialic acid was formed on the enzymes when chimeric enzymes contained the carboxyl terminal region of ST8Sia IV. However, the chimeric enzymes that have a short carboxyl terminal segment of ST8Sia IV showed less activity toward NCAM, even though they have strong activity in polysialylating themselves. In fact, chimeric enzymes containing the amino terminal region of ST8Sia IV demonstrated inhibitory effect on NCAM polysialylation by ST8Sia IV *in vitro*, suggesting the domain for NCAM recognition is distinct from the domain for polysialylation in the polysialyltransferase. We also found that presence of *N*-glycosylation affect ability of polysialic acid synthesis. Furthermore, our experiments clearly indicated that ST8Sia IV and chimeric enzymes can form polysialic acid on the molecules other than NCAM in cells and *in vitro*. (Supported by CA33895)

(291) **Hereditary Inclusion Body Myopathy: GNE and the Sialic Pathway in Muscle Cells.**

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Hereditary inclusion body myopathy (HIBM) is a unique group of neuromuscular disorders characterized by adult-onset, slowly progressive distal and proximal muscle weakness, and a typical muscle pathology with cytoplasmic "rimmed vacuoles" and cytoplasmic or nuclear inclusions composed of tubular filaments. The prototype of this disease, very frequent in the Jewish Persian community (with a prevalence of 1:1500), presents an unusual feature, the sparing of the quadriceps. While a single homozygous missense mutation (M712T) was identified in all Persian and other Middle Eastern Jewish patients in the gene encoding the enzyme UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE), different missense mutations in this same gene have been identified in quadriceps sparing HIBM cases diagnosed in isolated families of non-Jewish origin. GNE consists of two functional domains, an epimerase domain and a kinase domain. In contrast to the Persian Jewish patients which present a mutation in the kinase domain of GNE, most of the HIBM affected individuals of various ethnicities show a heterozygous composition of mutations, whereby all combinations of mutations among the domains, epimerase-epimerase, kinase-kinase and epimerase-kinase, were found. GNE catalyzes the first two steps of sialic acid biosynthesis in cells. Sialic acids are carbohydrates found on the cell surface where they mediate a lot of cellular functions, e.g. cell-cell or cell-matrix interactions. In spite of the key role of GNE in the biosynthesis of sialic acid, the pathological mechanism leading to HIBM remains unclear. To investigate this phenomenon, we have established muscle cultures from healthy and HIBM individuals and analyzed various steps in the sialic acid pathway. Constructs with GNE cDNAs carrying the "Middle Eastern" M712T mutation, localized in the kinase domain of the protein, as well as various different mutations identified in patients, at the different domains of the enzyme, were also overexpressed and the transfected cells analyzed. Results show that GNE mutations affect the sialic acid pathway in HIBM, with at least a 20% reduction of membrane bound sialic acid. Western blot analysis with the MAA lectin also suggests the a-2,3 hyposialylation of HIBM muscle glycoproteins. In order to elucidate if these sialylation defects solely can account for HIBM pathogenesis, we are searching for specific abnormally sialylated glycoproteins. Potential different mechanisms will also be discussed.

(292) **Defective Olfactory Development in β3GnT1 Null Mice**

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Sensory neurons of the nasal olfactory epithelium (OE) differ from neurons in the brain by their strong reactivity for the monoclonal antibody 1B2 which detects a terminal form of N-acetyllactosamine carbohydrate (LacNAc). 1B2+ olfactory sensory neurons (OSNs) reside in all topographic zones of the embryonic OE, but project axons that preferentially innervate ventromedial glomeruli in the olfactory bulb (OB) of the rostral cortex. This selective convergence suggests that LacNAc-modified glycans may influence axonal patterning between the peripheral OE and CNS, although direct evidence to support this has been limited. We show here that mice deficient for the glycosyltransferase β1,3-N-acetylglucosaminyl transferase-1 (β3GnT1) do not express 1B2-reactive LacNAc on OSNs neonatally, and they exhibit severe defects in postnatal olfactory development. Axonal projections to the OB are disorganized in null mice, with most axons failing to properly condense into discreet glomeruli. This deficit is paralleled by an increase in early postnatal OSN apoptosis and an activation of OSN progenitor cell proliferation. At later postnatal time points, an unexpected upregulation of LacNAc expression occurs on sensory axons of β3GnT1 null mice, possibly through compensatory expression of other glycosyltransferase activities. This increased expression, although concurrent with delayed axonal innervation to the OB, fails to restore normal glomerular organization. These results support an important role for LacNAc glycans in olfactory axon pathfinding, either through convergence, or sorting of axons within the OB, which is required for establishing proper connectivity with CNS targets and long term survival of OSNs. The effect of these defects on olfaction and behavior is currently under investigation.

(293) **Incomplete glycosylation of α-dystroglycan in Heriditary Inclusion Body Myopathy**

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Hereditary inclusion body myopathy (HIBM) is an adult onset autosomal recessive neuromuscular disorder characterized by slowly progressive myopathic weakness and atrophy. The myopathy is histologically characterized by muscle fiber degeneration and formation of rimmed vacuoles especially in atrophic fibers. HIBM is associated with mutations in the UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase gene (*GNE*). GNE is the rate limiting bi-functional enzyme catalyzing the first two steps of sialic acid biosynthesis. Loss of GNE activity due to *GNE* mutations impairs sialic acid production and interferes with proper sialylation of glycoconjugates. α-Dystroglycan is an essential component of the dystrophin-glycoprotein complex and is heavily glycosylated with some of the glycans O-linked via the sugar mannose (O-mannosylated). Kevin Campbell and others have recently shown abnormal dystroglycan-ligand interactions as the explanation for the pathogenic mechanism of several muscular dystrophies like Fukuyama's congenital muscular dystrophy (FCMD), Muscle-Eye-Brain disease (MEB) and Walker-Warburg syndrome. The clinical findings of muscular dystrophy in HIBM suggested a perturbation of the α-dystroglycan–ligand interaction in the pathogenesis of HIBM. Analysis of the glycosylation of α-dystroglycan was explored by immunostaining of skeletal muscle tissue from three HIBM patients. This showed a dramatically decreased immunoreaction to both VIA4-1 and IIH6 α-dystroglycan antibodies. These antibodies are thought to specifically detect the O-mannosylated glycoconjugates on α-dystroglycan. Antibodies to β-dystroglycan and laminin α-2 showed a normal pattern in HIBM muscle when compared to control muscle. Western blot analysis performed on muscle tissue from the same patients showed decreased signals for VIA4-1 and IIH6 compared to control, while β-dystroglycan signals were normal, as expected.

What is not clear is why similar findings in the glycopathology of α-dystroglycan can lead to both severe childhood onset diseases, such as FCMD or MEB, and an adult onset disorder, HIBM. Perhaps the residual activity of *GNE*, known to be present in affected individuals, leads to a large enough sialic acid pool to spare individuals with HIBM from an earlier onset of skeletal muscle symptoms. We also know that the serum transferrin isoforms, reflecting N-linked glycosylation, are normal in patients with HIBM. This would suggest that other glycoconjugates (N-linked , O-GalNAc linked) might be preferentially glycosylated over the very rare O-mannosylated glycans. Perhaps when the body has a shortage of sialic acid, the O-mannosylated glycoproteins, like muscle α-dystroglycan, will be not be sialylated as efficiently. The fact that the glycosylation patterns in α-dystroglycan are tissue-specific increases the complexity of this dynamic glycosylation process. Understanding the function and regulation of the O-linked mannose pathway is essential for development of diagnostic tests and therapies for HIBM and other muscular dystrophies with similar pathology.

(294) **C6 Glioma Invasion into Corpus Callosum Is Facilitated by Polysialylation of NCAM**

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Tumor of central nervous system accounts for 2.3% of cancer-related deaths. The majority of these tumors is either glioblastoma multiforme or anaplastic astrocytoma, and few of these patients will ultimately be cured of their disease. Since we expect that cell adhesion molecule of glioma cells may play a role in malignancy, we investigated expression of Neural Cell Adhesion Molecule (NCAM) and polysialic acid. First we examined 50 human glioma specimens and detected tumor of 30 cases expressing NCAM, among them 9 cases of relatively advanced astrocytoma also expessed polysialic acid. Since polysialic acid is known to attenuate NCAM adhesive property, we then examined the effect of polysialylation on C6 glioma cell migration in the brain. We established ST8Sia II polysialyltransferase stable transfectant of C6 rat glioma cell line and performed stereotaxic injection of those C6 cells into the left half sphere of wild type mice brain. Three weeks after injection, we found that the parent C6 cells spread extensively in the left half of brain, but tumor invasion was prevented at the site of corpus callosum. On the other hand, C6 cells expressing polysialic acid invaded aggressively, going to cross corpus callosum. We performed similar injection experiments with Myelin Associated Glycoprotein (MAG) deficient mice and NCAM deficient mice. Since MAG is known to be expressed on myelin sheath which is concentrated at corpus callosum and also known to have a function as a sialic acid binding protein, we expected to see perturbation of C6 cell invasion character. Interestingly, in MAG deficient mice brain, not only control C6 cells but also polysialylated C6 cells invasion was prevented at corpus callosum. By contrast, in NCAM deficient mice we could observe invasion of tumor at corpus callosum even for control C6 cells. To support these findings above, *in vitro* transwell cell migration assay was performed. NCAM or MAG molecule alone did not induce C6 cell migration in transwell assay, however with existence of fibronectin, NCAM attenuated and MAG facilitated migration of C6 cells. These results indicate that in wild type condition MAG on corpus callosum is facilitating C6 cells to invade, but NCAM-NCAM homophilic interaction between C6 cells and corpus callosum inhibits invasion of C6 cells. When NCAM on C6 cells is polysialylated, NCAM-NCAM interaction is reduced and tumor cells increase migration activity. Work is in progress to support this finding using cell binding assay.

(295) **Carbohydrate Assays for Characterization and Release of Therapeutic Glycoproteins**

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Selection of assays and acceptance criteria for release and stability testing of recombinant glycoproteins is a process which evolves through product development. Choice of assays and acceptance criteria is determined through a combination of characterization studies with preclinical and clinical experience. Relevant features of glycosylation determined through clinical experience should ultimately drive selection of tests and limits. A discussion of assay characteristics for a range of assays (mass spectrometric and chromatographic) available for glycoprotein characterization will be presented. Validation of a fluorescent-labeled, anion-exchange assay use for release and stability testing of a sialylated glycoprotein will also be presented.

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