PROGRAM AND ABSTRACTS FOR THE 2013 ANNUAL CONFERENCE OF THE SOCIETY FOR GLYCOBIOLOGY

November 17–20, 2013
St. Petersburg, Florida, US.
## Annual Conference of the Society for Glycobiology

### Program

#### ANNUAL CONFERENCE of the SOCIETY FOR GLYCOBIOLOGY

**November 17–20, 2013**  
Vinoy Renaissance St. Petersburg Resort & Golf Club  
St. Petersburg, FL

**Christopher M. West**, Program Chair and President  
*University of Oklahoma Health Sciences Center*

### Program Overview

<table>
<thead>
<tr>
<th>Date</th>
<th>Time</th>
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<tr>
<td><strong>Sunday, November 17</strong></td>
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</table>
| 8:00 am – 5:00 pm | **Junior Scientist Pre-Conference** (SFG Networking and Career Mentoring Pre-Conference)  
Chairs: Richard and Heather Steet, *University of Georgia CCRC* |  |
| 8:30 am – 5:30 pm | **Satellite Symposium I: Glycoprotein Technologies**  
Chairs: Wesley Wang, *Amgen*; Sam Tep, *Biogenidec* |  |
| 9:00 am – 4:00 pm | **Satellite Symposium II: Consortium for Functional Glycomics (CFG): Development and Application of Transformative Technologies in Glycobiology**  
Chairs: Brian A. Cobb, *Case Western Reserve University College of Medicine*; Christine Szymanski, *Alberta Glycomics Centre, University of Alberta* |  |
| 7:00 – 7:10 pm | **Conference Opening Remarks**  
Christopher M. West, President, Society for Glycobiology |  |
| 7:10 – 8:25 pm | **Keynote Lecture/Session I: Glycoproteomics, Polysaccharides, and Glycomics**  
Chair: Anne Dell, *Imperial College London* |  |
| 8:25 – 9:05 pm | **Rosalind Kornfeld Award for Lifetime Achievement in Glycobiology Award Presentation**  
Chair: Naoyuki Taniguchi, *RIKEN*  
2013 recipient: **Carlos Hirschberg**, *Boston University Goldman School of Dental Medicine* |  |
| 9:05 – 10:00 pm | **Welcome Reception** |  |
| **Monday, November 18** | |  |
| 8:30 – 10:00 am | **Session II: Developmental Glycobiology**  
Chair: Michael Tiemeyer, *University of Georgia CCRC* |  |
| 10:30 am – 12:25 pm | **Session III: Glycosylation, the Secretory Pathway, and Biosynthesis**  
Chair: Richard Cummings, *Emory University* |  |
| 2:00 – 4:00 pm | **Poster Session I and Exhibits** |  |
| 4:00 – 5:55 pm | **Session IV: Metabolism, Recognition, and Signaling**  
Chair: Gerald Hart, *Johns Hopkins University* |  |
| **Tuesday, November 19** | |  |
| 8:30 – 10:00 am | **Session V: Inhibitors**  
Chair: Ole Hindsgaul, *Carlsberg Laboratory* |  |
| 10:30 am – 12:25 pm | **Session VI: Glycobiology of Infectious Diseases**  
Chair: Anant Menon, *Weill Cornell Medical College* |  |
| 2:00 – 4:00 pm | **Poster Session II and Exhibits** |  |
| 3:30 – 4:00 pm | **Grant Funding Agency Workshop** (optional) |  |
| 4:00 – 4:30 pm | Business Meeting (open to all attendees) |  |
| 4:45 – 5:30 pm | **Karl Meyer Award Lecture**  
Chair: Naoyuki Taniguchi, *RIKEN*  
2013 recipient: **Markus Aebi**, *Swiss Federal Institute of Technology* |  |
<p>| 6:30 – 11:00 pm | <strong>Pre-Banquet Social Gathering &amp; Banquet</strong> |  |</p>
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<tr>
<th>Time</th>
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<tr>
<td>8:30 – 10:00 am</td>
<td>Session VII: IGO Guest Session: Cancer Glycobiology</td>
<td>Jianing Zhang, <em>Dalian Institute of Technology</em></td>
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<tr>
<td>10:30 am – 12:25 pm</td>
<td><strong>Session VIII: Vascular Glycobiology</strong></td>
<td>Jeff Esko, <em>University of California at San Diego</em></td>
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<td>2:00 – 4:00 pm</td>
<td><strong>Poster Session III and Exhibits</strong></td>
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<tr>
<td>4:00 – 5:40 pm</td>
<td><strong>Session IX: Human Disease</strong></td>
<td>Linda Baum, <em>UCLA</em></td>
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<tr>
<td>5:40 – 5:45 pm</td>
<td><strong>Closing remarks</strong></td>
<td>Christopher M. West, President, Society for Glycobiology</td>
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</table>
PROGRAM OVERVIEW

SUNDAY, NOVEMBER 17

7:30 am – 7:00 pm
Registration Desk (Vinoy Foyer)

8:30 am – 6:00 pm
Exhibit Set-up (Palm Court Ballroom/foyer)

8:00 am – 5:00 pm
New! Junior Scientist Pre-Conference (SFG Networking and Career Mentoring Pre-Conference)
Plaza Ballroom A
Registration: $50
Chairs: Richard and Heather Steet, University of Georgia CCRC
Open to Graduate Students and Post-Docs (0-5 Years experience) in GlycoScience
In an effort to bring young scientists together to discuss their current research, build informal networks with their peers and gain career mentorship, the Society for Glycobiology hosts this 1-day pre-conference. Organized and supported by a faculty of eight leading scientists from the Society, this pre-conference will be centered around scientific talks and poster presentations chosen from conference abstracts submitted by graduate students and post-docs. In addition, prospective talks will be given by glycoscientists working in diverse areas of clinical and basic research to provide a platform for discussing the many career paths and opportunities available to emerging Ph.D. scientists.

8:30 am – 5:30 pm
Satellite Symposium I: Glycoprotein Technologies
Plaza Ballroom B
Registration: $50
Chairs: Wesley Wang, Amgen; Sam Tep, Biogeniedc
The scope of this session encompasses all areas of glycoprotein technologies, ranging from bioprocess control through downstream analytical/characterization techniques. Functional studies and Structure-Activity-Relationship (SAR) studies, efforts regarding the challenges in the production of biosimilars and follow-on biologics, as well as efforts in the characterization and analysis of O-glycosylation, are all included.

9:00 am – 4:00 pm
Satellite Symposium II: Consortium for Functional Glycomics (CFG):
Development and Application of Transformative Technologies in Glycobiology
Vinoy Grand Ballroom
Registration: $50
Chairs: Brian A. Cobb, Case Western Reserve University College of Medicine; Christine Szymanski, Alberta Glycomics Centre, University of Alberta
This satellite symposium is focused upon recent technological advances in glycomics, and how these novel tools have been successfully applied to better understand critical biological processes and diseases. Upon the completion of this symposium, attendees will be aware of these emerging technologies as well as collaborative and service-based opportunities to apply them to their particular research interests.

7:00 – 7:10 pm
Conference Opening Remarks (Vinoy Grand Ballroom)
Christopher M. West, President, Society for Glycobiology
7:10 – 8:25 pm
Keynote Lecture/Session I: Glycoproteomics, Polysaccharides, and Glycomics (Vinoy Grand Ballroom)
Chair: Anne Dell, Imperial College London

Time Abstract Number
7:10 The importance of electron transfer mass spectrometry in advancing our understanding of structural glycobiology; Al Burlingame; University of California San Francisco. *Plenary Lecture sponsored by MCP/ASBMB .......................... 1
7:35 NextGen sequencing for the identification of glycosyltransferases involved in plant cell wall polysaccharide biosynthesis; Markus Pauly¹, Alex Schultink¹, Jacob Jensen²; ¹UC Berkeley; ²Michigan State University ......................... 2
8:00 Glycomics and glycoproteomics- providing new biological insights; Stuart Haslam, Maria Panico, Howard Morris, Anne Dell; Imperial College London ................................................................. 3

8:25 – 9:05pm
Rosalind Kornfeld Award for Lifetime Achievement in Glycobiology Award Presentation (Vinoy Grand Ballroom/Foyer)
Chair: Naoyuki Taniguchi, RIKEN
2013 recipient: Carlos Hirschberg;
Boston University Goldman School of Dental Medicine

9:05 – 10:00pm
Welcome Reception (Palm Court Ballroom/Foyer)

MONDAY, NOVEMBER 18

7:30 am – 4:00 pm
Registration Desk (Vinoy Foyer)
Speaker Ready Room (Taylor Room)

7:30 – 8:30am
Continental Breakfast *Sponsored by Promega Corporation
Palm Court Ballroom/Foyer

8:30 – 10:00am
Session II: Developmental Glycobiology (Vinoy Grand Ballroom)
Chair: Michael Tiemeyer, University of Georgia CCRC

Time Abstract Number
8:30 Role of O-glucose glycans in animal development; Hamed Jafar-Nejad², Amanda R. Haltom¹, Tom V. Lee², Beth Harvey³, Jessica Leonard², Robert S. Haltiwanger¹; ¹University of Texas Health Science Center; ²Baylor College of Medicine; ³Stony Brook University......................................................... 4
8:55 The in vivo function of Drosophila CMP-sialic acid synthetase; Hilary Scott, Rafique Islam, Courtney Caster, Mark J. Zoran, Vladislav M. Panin; Texas A&M University *selected talk-poster #48 .............................................................. 5
9:00 O-fucosylation and development; Robert Haltiwanger, Deepika Vasudevan, Christina Leonhard-Melief, Stony Brook University ................................................................. 6
9:25 Glycosyltransferases as systemic regulators of blood cell development; Joseph Lau; Roswell Park Cancer Institute ....... 7
9:50 Possible role of cathepsin-mediated growth factor activation in the pathogenesis of mucolipidosis II; Megan Aarnio, Heather Flanagan-Stee, Aaron Petrey, Richard Steet; CCRC University of Georgia *selected talk-poster #108........................................................... 8
9:55 A murine knockout of the human diabetes susceptibility locus Oga contributes to acute and chronic nutrient sensing defects; Dona Love, Chithra Keembiyahety, Oksana Gavrilova, Marcy Comly, Katryn Harwood, John Hanover; NIDDK, NIH *selected talk-poster #108........................................................... 9
10:00 – 10:30 am
Coffee Break (Tea Garden)

10:30 am – 12:25 pm
Session III: Glycosylation, the Secretory Pathway, and Biosynthesis
(Vinoy Grand Ballroom)
Chair: Richard Cummings, Emory University

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10:30 – 10:55
Identification of a GlaC transferase involved in biosynthesis of glycosyl inositol phosphorylceramide sphingolipids in plants; **Jochen Zimmer**, University of Virginia ........................................ 10

10:55 – 11:00
GDP-Mannose transport in Cryptococcus neoformans is mediated by two distinct proteins and is dispensable for viability; Zhuo Wang, Matthew Williams, Tamara Doering; Washington University School of Medicine *selected talk-poster #49 ................................. 11

11:05 – 11:10
Characterizing a missense mutation in O-GlcNAc transferase that segregates with disease in a family with X-linked Intellectual Disability; **Krithika Vaidyanathan**, Tejasvi Niranjan, Melanie May, Rebecca Rose, Chin Fen Teo, Sindhu Prabakaran, Robert Bridger, Emil Alexov, Tao Wang, Charles Schwartz, Lance Wells; *CCRC, University of Georgia; Johns Hopkins-McKusick-Nathans Institute of Genetic Medicine; Greenwood Genetic Center; Clemson University *selected talk-poster #109 .................................................. 12

11:45 – 11:50
Pathways and regulation of IgAl O-glycosylation in an autoimmune disease, IgA nephropathy; **Jan Novak**, Jan Novak, Kazu Takahashi, Hitoshi Suzuki, Milan Rasku, Tyler Stewart, Koshi Yamada, Colin Reilly, Hiroyuki Ueda, Zhi-Qiang Huang, Audra Hargett, Stacy Hall, Milada Stuchlova Horynova, Zina Moldoveanu, Jiri Mestecky, Bruce A. Julian, Ali G. Gharavi, Krzysztof Kiryluk, Mathew B. Renfrow; University of Alabama at Birmingham; *Columbia University University of Nebraska Medical Center *selected talk-poster #150 .................................................. 13

12:10 – 12:15
Differential Golgi targeting of βgalactosidase:α2-3sialyltransferase-1 and core 2 N-acetylgalcosaminyltransferase-L1 in advanced prostate cancer cells; **Pi Wan Cheng**, Armen Petrosyan; VA Nebraska Western Iowa Health Care System and University of Iowa Roy J. and Lucille A. Carver College of Medicine *Young Investigator Award sponsored by BBA General Subjects ........................................ 14

12:20 – 12:25
GOLPH3 regulates integrin-mediated biological functions via up-regulation of sialylation; **Jian Guo Gu**, Tomoya Isaji, Sanghun Im, Tomohiko Fukuda; Tohoku Pharmaceutical University *selected talk-poster #165 ............ 15

12:25 – 2:00 pm
Lunch on your own

12:25 – 2:00 pm
Glycobiology Editorial Board Meeting Lunch (Invitees only) (Thomasson Room)

2:00 – 4:00 pm
Poster Session I and Exhibits
Coffee break provided
Palm Court Ballroom/Foyer
4:00 – 6:00 pm
Session IV: Metabolism, Recognition, and Signaling
Vinoy Grand Ballroom
Chair: Gerald Hart, Johns Hopkins University

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<tr>
<td>4:00</td>
<td>Non-lysosomal degradation pathway for N-glycans; cases in yeast and mammalian cells; Tadashi Suzuki, Rachel Kubiak, James Thoden; RIKEN Global Research Cluster, Japan</td>
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<td>4:25</td>
<td>Discovery of novel toxin binding partners using photocrosslinking sialic acid; Jennifer Kohler, Amberlyn M. Wands, Akiko Fujita, Janet E. McCombs; University of Texas Southwestern Medical Ctr.</td>
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<td>4:50</td>
<td>Structural insights into novel deoxysugar biosynthesis in bacteria; Hazel Holden, University of Wisconsin–Madison</td>
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<td>5:15</td>
<td>Further characterization of endogenous mouse lung proteins bearing glycan counter-receptors for Siglec-F; Toshihiko Katoh¹, Takumi Kiwamoto², Christopher Evans³, Mary Brummet², Sherry Hudson², Zhou Zhu², Bruce Bochner², Michael Tiemeyer¹; ¹CCRC, University of Georgia; ²Johns Hopkins University School of Medicine; ³University of Colorado School of Medicine *selected talk-poster #151</td>
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<td>5:20</td>
<td>Context dependent glycan recognition through the next generation of glycan microarray; Geert-Jan Boons, University of Georgia CCRC</td>
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<td>5:45</td>
<td>Reductionist model systems to probe biological and molecular mechanisms of O-GlcNAc signalling; Daniel van Aalten, University of Dundee *selected talk-poster #110</td>
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<td>5:50</td>
<td>O-GlcNAc regulated arginine methylation: a novel paradigm in survival signaling; Natasha Zachara, Albert Lee, Kamau Fahie, Roger Henry, Devin Miller; Johns Hopkins University School of Medicine *selected talk-poster #77</td>
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<td>5:55</td>
<td>Increased O-GlcNAcylation of Mitochondrial Proteins Directly Contributes to ROS Production in Diabetes; Junfeng Ma, Ting Liu, Brain O’Rouke, Gerald Hart; Johns Hopkins University *selected talk-poster #111</td>
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TUESDAY, NOVEMBER 19

7:30 am – 4:00 pm
Registration Desk
( Vinoy Foyer)
Speaker Ready Room
Taylor Room

7:30 – 8:30 am
Continental Breakfast *Sponsored by Galen Laboratory Supplies & Iduron
Palm Court Ballroom/Foyer

8:30 – 10:00 am
Session V: Inhibitors
Vinoy Grand Ballroom
Chair: Ole Hindsgaul, Carlsberg Laboratory

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<tr>
<td>8:30</td>
<td>From carbohydrates to glycomimetics - is there a general recipe?; Beat Ernst, University of Basel</td>
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<td>8:55</td>
<td>Sweet Contacts - Guiding inhibition of carbohydrate-protein interactions with NMR; Thomas Peters; University of Luebeck</td>
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<tr>
<td>9:20</td>
<td>Rivipansel (GMI-1070), a glycomimetic pan-selectin antagonist, inhibits activation and thrombosis in animal models as well as functional biomarkers in sickle cell patients; John Magnani¹, John Patton¹, Henry Flanner¹, Daniel Myers², TedWun³, Helen Thackray¹, Scott Simon³; ¹GlycoMimetics, Inc; ²Univ of Michigan; ³Univ of California Davis; *Univ of California Davis School of Medicine *selected talk-poster #122</td>
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<tr>
<td>9:25</td>
<td>Glycan based inhibitors of receptors and enzymes; Bernd Meyer, Katrin Schaefer, Felix Niemeyer, Wei Nien Liao, Moritz Waldmann, Dennis Wilhelm, Miriam Koetzler, Patrizia Leccese; University of Hamburg *selected talk-poster #123</td>
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9:30 Metabolic inhibitors of glycosyltransferases as tools for glycobiology; David Vocadlo, Scott Yuzwa, Xiaoyang Shan, Nevena Cekic, Lehua Deng, Julia Heinonen, Keith Stubbs, Tracey Gloster, Yanping Zhu; Simon Fraser University ................................................................. 33
9:55 Detection of glycosyltransferase activities with homogenous bioluminescent UDP detection assay; Hicham Zegzouti, Laurie Engel, Gediminas Vidugiris, Said Goueli; Promega Corporation *selected talk-poster #19 .................. 34

10:00 – 10:30 am
Coffee Break
Mezzanine/Terrace

10:30 – 12:30 pm
Session VI: Glycobiology of Infectious Diseases
Vinoy Grand Ballroom
Chair: Anant Menon, Weill Cornell Medical College

Time Abstract Number
10:30 The expanding world of bacterial protein O-linked glycosylation; Mario Feldman, Alberta Glycomics Centre-University of Alberta ................................................................. 35
10:55 N-Glycan biosynthesis and utilization in African trypanosomes; James Bangs, University at Buffalo School of Medicine ................................................................. 36
11:20 The oocyst walls of Toxoplasma have an inner layer of β-1,3-glucan (like fungi) and an outer layer of acid-fast lipids (like mycobacteria); Edwin M. Motari1, G. Guy Bushkin2, Jitender P. Dubey3, Catherine E. Costello4, Phillips W. Robbins1, John Samuelson1; 1Boston University School of Dental Medicine; 2Massachusetts Institute of Technology; 3United States Department of Agriculture; 4Boston University School of Medicine *selected talk-poster #128 ................. 37
11:25 Galectin-3 is a damage-associated molecular pattern molecule, which facilitates neutrophil recruitment as an innate immune response to a parasitic protozoan cutaneous infection; Sachiko Sato, Pampa Bhaumik, Guillaume St-Pierre, Valérie Milot, Julie-Christine Levesque, Christian St-Pierre; Laval University; *selected talk-poster #129 ............................. 38
11:30 Immune regulation by glycans; Brian Cobb, Jenny L. Johnson, Mark B. Jones; Case Western Reserve University School of Medicine ................................................................. 39
11:55 Fungal chitin and the immunological end game; Neil A.R. Gow1, Jeanette Wagener1, R.K.S. Malireddi2, Megan D. Lenardon1, Martin Koberle3, Simon Vautier1, Donna M. MacCallum1, F.J. Alvarez1, Tilo Biedermann3, Martin Schaller1, Mihai G. Neta4, T.D. Kanneganti2, Gordon D. Brown1, Alistair J.P. Brown1; University of Aberdeen, Aberdeen Fungal Group; 2St. Jude Children’s Research Hospital; 3Eberhard Karls University Tübingen; 4Radboud University Nijmegen Medical Center .............................. 40
12:20 Zebrafish galectins bind to the infectious hematopoietic necrosis virus (IHNV) glycoprotein and modulate adhesion to the host cell surface; Mihai Nita-Lazar1, Chinnarajan Ravindran2, Barbara Giomarelli1, Marta Pasek1, Shawn Jackson1, Ana de las Heras Sanchez1, Hafiz Ahmed1, Arun Ammayappan3, Vikram Vakharia3, Anne Dell1, Stuart M. Haslam4, Gang Wu1, Chiguang Feng1, Gerardo R. Vasta1; 1University of Maryland School of Medicine, Institute of Marine and Environmental Technology; 2National Institute of Oceanography (CSIR); 3University of Maryland Baltimore County; 4Imperial College *selected talk-poster #130 ................................................................. 41
12:25 Human milk glycans are potential receptor decoys during neonatal rotavirus infection; Ying Yu1, Yi Lasanajak1, Xuezheng Song1, Liya Hu2, Sasirekha Ramani2, David J. Ashline3, B.V. Venkataram PrasadF, Mary K. Estes2, Vernon N. Reinhold1, Richard D. Cummings3, David F. Smith1; 1Emory University School of Medicine; 2Baylor College of Medicine; 3University of New Hampshire *selected talk-poster #131 ................. 42

12:30 – 2:00 pm
Lunch on your own

12:30 – 2:00 pm
SFG Board Meeting Lunch (Invitees only)
Thomasson Room
### 2:00 – 4:00 pm
**Poster Session II and Exhibits**
Coffee break provided
*Palm Court Ballroom/Foyer*

### 3:30 – 4:00 pm
(optional) Grant Funding Agency Workshop (Representatives from NIH, NSF, and more)
*Vinoy Ballroom*

### 4:00 – 4:30 pm
Business Meeting (open to all attendees)
*Vinoy Ballroom*

### 4:45 – 5:30 pm
**Karl Meyer Award Lecture**
*Vinoy Grand Ballroom/Foyer*
Chair: Naoyuki Taniguchi, RIKEN
2013 recipient: **Markus Aebi**, *Swiss Federal Institute of Technology*

### 6:30 – 7:00 pm
**Pre-Banquet Social Gathering**
*Vinoy Grand Ballroom Foyer*

### 7:00 – 11:00 pm
**Banquet**
*Vinoy Grand Ballroom*
Nominal fee. Extra tickets for guests may be ordered.

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**WEDNESDAY, NOVEMBER 20**

### 7:30 – 4:00 pm
**Registration Desk**
*Vinoy Foyer*
**Speaker Ready Room**
*Taylor Room*

### 7:30 – 8:30 am
**Continental Breakfast** *Sponsored by Galen Laboratory Supplies & Iduron*
*Palm Court Ballroom/Foyer*

### 8:30 – 10:00 am
**Session VII: IGO Guest Session: Cancer Glycobiology**
*Vinoy Grand Ballroom*
Jianing Zhang, *Dalian Institute of Technology*

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<th>Time</th>
<th>Abstract Number</th>
<th>Title</th>
<th>Speaker/Institution</th>
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<tr>
<td>8:30</td>
<td>43</td>
<td>Roles of heparan sulfate in normal and cancer stem cells of intestine; <strong>Minoru Fukuda</strong>;</td>
<td>Sanford-Burnham Medical Research Institute</td>
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<td>8:50</td>
<td>44</td>
<td>Intratumoral hypoxia induces sialyl-Tn antigen expression and facilitates tumor metastasis;</td>
<td><strong>Kazuaki Ohtsubo</strong>; Kumamoto University</td>
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</table>
9:10 Harnessing novel biomarkers of human embryonic stem cells for cancer diagnosis and therapy; **John Yu**; Chang Gung Memorial Hospital at Linkou, Taoyuan

9:30 Heparan sulfate is required for prostate cancer initiation and progression; **Lianchun Wang**, Xuanyang Li, Alison Nairn, Tamas Nagy, Fen Wang, Yu Yamaguchi, Kelley Moremen; Complex Carbohydrate Research Center and University of Georgia; University of Georgia; Texas A&M Health Science Center; Sanford-Burnham Institute for Medical Research

9:45 O-GlcNAc modification of ribosomal RACK1 enhances hypoxia-induced epithelial–mesenchymal transition in hepatocellular carcinoma; **Yuanyuan Ruan**, Jianxin Gu; Fudan University

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**10:00 – 10:30 am**

Coffee Break
Palm Court Ballroom/Foyer

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**10:30 am – 12:30 pm**

Session VIII: Vascular Glycobiology
Vinoy Grand Ballroom
Chair: Jeff Esko, University of California at San Diego

**Time** | **Abstract Number** | **Abstract**
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10:30 | 48 | Syndecan-coupled receptor kinase signaling in angiogenesis; **Alan Rapraeger**, University of Wisconsin-Madison

10:55 | 49 | Glycosylation in the metabolic origins of common grievous disease; **Jamey Marth**, Won Ho Yang, Peter Aziz, Javier Ochoa-Reparaz; University of California at Santa Barbara

11:20 | 50 | Galectin-3 disruption impaired melanoma associated angiogenesis and reduced VEGF secretion from macrophages stimulated by TGFβ1; **Roger Chammas**, Camila Machado, Luciana Sousa Andrade, Verônica Teixeira, Fabrício Costa, Camila Melo, Sofia dos Santos, Suely Nonogaki, Fu-Tong Liu, Emerson Bernardes, Anamaria Camargo; 1Faculdade de Medicina da Universidade de São Paulo; 2Instituto do Câncer do Estado de São Paulo; 3Northwestern University's Feinberg School of Medicine; 4Instituto Adolfo Lutz, São Paulo; 5Academia Sinica; 6Ludwig Institute for Cancer Research, São Paulo Branch *selected talk-poster #166*

11:25 | 51 | The role of glycansynthesis and degradation in platelet birth and death; **Karin Hoffmeister**, Brigham and Women’s Hospital/Harvard Medical School

11:50 | 52 | Heparosan polysaccharide for drug delivery: a biosuperior alternative to PEGylation; **Paul DeAngelis**, University of Oklahoma Health Sciences Center

12:15 | 53 | Ovarian tumor cell survival mechanisms regulated by ST6Gal-I sialyltransferase; **Susan L. Bellis**, Matthew Schultz, Charles N. Landen; University of Alabama at Birmingham *selected talk-poster #167*

12:20 | 54 | A novel neu1 sialidase and matrix metalloproteinase-9 crosstalk regulates epidermal growth factor receptor and its targeted translation in pancreatic cancer; **Myron Szewczuk**, Alanna M. Gilmour, Samar Abdulkhalek, Timothy S.W. Cheng, Farah Alghamdi, Preethi Jayanth, Leah K. O’Shea, Olivia Geen, Luis A. Arvizu; Queen’s University *selected talk-poster #168*

12:25 | 55 | Mucin-type O-glycoprotein podoplanin is essential for maintaining high endothelial venule integrity by interacting with platelet CLEC-2; **Lijun Xia**, Jianxin Fu, Brett H. Herzog; Oklahoma Medical Research Foundation; University of Oklahoma Health Sciences Center *selected talk-poster #180*

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**12:30 – 2:00 pm**

Lunch on your own

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**2:00 – 4:00 pm**

Poster Session III and Exhibits
Coffee break provided
Palm Court Ballroom/Foyer
### 4:00 – 5:40 pm
**Session IX: Human Disease**

Vinoy Grand Ballroom  
Chair: Linda Baum, UCLA

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<td>4:00</td>
<td>Insight into UDP-GlcNAc : lysosomal enzyme N-acetylglucosamine-1-phosphotransferase function/localization via analysis of mutations in Mucolipidosis II/III; <strong>Stuart Kornfeld; Washington University School of Medicine</strong> .......... 56</td>
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<tr>
<td>4:25</td>
<td>The Big Bang: an expanding universe of human glycosylation disorders; <strong>Hudson Freeze; Sanford-Burnham Medical Research Institute</strong> ................................................................................................................................................. 57</td>
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Closing remarks  
Vinoy Grand Ballroom  
Christopher M. West, President, Society for Glycobiology
MONDAY, NOVEMBER 18

Poster Session I: 2:00 – 4:00 pm (Palm Court Ballroom/Foyer)

Poster Topics:

Analytical Services;
Glycoproteomics, Polysaccharides, and Glycomics; Glycotechnologies; Developmental Glycobiology;
Glycosylation, the Secretory Pathway, and Biosynthesis; Glycosyltransferase Biochemistry

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Analytical Services

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1 Analytical Services and Trainings at the Complex Carbohydrates Research Center; Roberto Sonon, Mayumi Ishihara, Zhirui Wang, Christian Heiss, Ian Black, Tina Thomas, Radhna Naran, Stephanie Archer-Hartman, Parastoo Azadi; University of Georgia ......................................................... 60

2 Strategies for Glycomics and Glycosaminoglycan Analysis as part of Analytical Services at the Complex Carbohydrate Research Center; Stephanie Archer-Hartmann, Mayumi Ishihara, Christian Heiss, Guerard Byrne, Christopher McGregor, Farooq Ahmed, Jiahua Xie, Tarun Saxena, Ravi Bellamkonda, Tobey MacDonald, Parastoo Azadi; Complex Carbohydrate Research Center, UGA, Athens, GA, Institute of Cardiovascular Science, University College London, London, UK, Department of Pharmaceutical Sciences, Biomannufacturing research Institute & Technology Enterprise, North Carolina Central University, Durham, NC, Laboratory for Neuroengineering, Georgia Institute of Technology, Atlanta, GA, School of Medicine, Emory University, Atlanta, GA ......................................................... 61

3 Repository of recombinant expression constructs for mammalian glycosylation enzymes: production of glycosyltransferases and glycoside hydrolases in mammalian cells; K.W. Moremen, H. Moniz, A. Ramiah, L. Meng, Z. Gao, A.V. Nairn, Y. Xiang, M. dela Rosa, D. Jarvis, J. Steel, J. LaBaer; Complex Carbohydrate Research Center, University of Georgia, Athens, GA, Department of Molecular Biology, University of Wyoming, Laramie, WY, Biodesign Institute, Arizona State University, Tempe, AZ ......................................................... 62

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**Program Overview**

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**December 15, 2011**

**Poster Session II: 2:00 – 4:00 pm (Palm Court Ballroom/Foyer)**

**Poster Topics:**
- Glycoimmunology & Inflammation; IGO Guest Session: Cancer Glycobiology;
- Vascular Glycobiology; Human Genetic Diseases; Late Breaking Posters

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165 Glycosaminoglycans Recovered from Alzheimer’s Disease Tissue Culture; Stephanie A Archer-Hartmann1,
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171 Surface coupling of P-selectin glycoprotein ligand-1 onto mesenchymal stem cells enables leukocyte-like
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Kitazume2, Akihiko Gemma2, Kozui Kida2, Naoyuki Taniguchi3; 1Institute of Biological Chemistry, Academia
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and Department of Internal Medicine, Nippon Medical School, 3Systems Glycobiology Research Group, Global
Research Cluster, RIKEN, 4Department of Internal Medicine, Nippon Medical School

173 Lactosylceramide induces hypertrophy in cardiomyocytes; Sumita Mishra, Puchong Thirawatananond, Subroto
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174 Inhibiting Glycosphingolipid glycosyltransferase activity Prevents Cardiac hypertrophy in apoE−/−mice fed
western diet and C57 Bl-6 mice subject to trans-aortic constriction; Subroto Chatterjee, Djahida Bedja, Sumita
Mishra, David Kass, Christine Amuzie, Mark Renehan; Johns Hopkins Medical Institutions
(1) The importance of electron transfer mass spectrometry in advancing our understanding of structural glycobiology

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UDP-GlcNAc (β-N-acetylglucosamine) is an abundant cellular cofactor that represents the integration of intracellular metabolic and nutrient sensing pathways with dynamic covalent posttranslational regulation of a large number of cytosolic and nuclear proteins. Two enzymes are involved: O-GlcNAc-transferase (OGT) and O-GlcNAcase(OGA). Current research has implicated protein O-GlcNAcylation in many cellular processes including the central nervous system, chromatin remodeling complexes, regulation of stem cell 'stemness' and a wide variety of human diseases including sleep disorders, diabetes, Alzheimer and cancer. For more than a decade we have sought to develop experimental strategies that would permit both the rapid, robust enrichment of O-GlcNAcylated proteins and the unambiguous assignment of sites of protein modification. This work has established that the recently developed electron capture and transfer energy deposition techniques are particularly effective at retaining O-GlcNAc moieties in peptide sequence ion series, thus enabling unambiguous assignment of sites of modification on serine and threonine residues. We have refined our protocol for enrichment of native O-GlcNAcylated peptides based on use of the lectin, wheat germ agglutinin (WGA). In addition we have carried out a comprehensive analysis of N-and O-linked glycosylation enriched by our WGA strategy in addition to O-GlcNAc modified peptides. For protein O-GlcNAc per se, we have focused efforts on deciphering the site specific functions of O-GlcNAcylation in several biological settings including the synapse, regulation of the circadian clock and stem cell pluripotency. Studies of the murine synapse have explored a sequential enrichment strategy for both O-GlcNAcylation and phosphorylation from the same biological preparation. This work has revealed some 1600 sites of O-GlcNAcylation and over 16,000 sites of phosphorylation. In addition, we have shown dynamic involvement of O-GlcNAcylation with phosphorylation of a region of PER2 known to regulate human sleep phase. Finally we have established the role of sox2 O-GlcNAcylation in the efficiency of somatic cell reprogramming to iPSCs. This presentation will outline our current understanding of O-GlcNAc function and its relation to S/T phosphorylation. Financial support was provided by the NIH NIGMS Grant P41GM103481, the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, and the Howard Hughes Medical Institute (to ALB).

(2) NextGen sequencing for the identification of glycosyltransferases involved in plant cell wall polysaccharide biosynthesis

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The polysaccharides present in the plant cell wall represent the dominant carbon sequestration system on the planet as their production capacity on serine and threonine residues. We have refined our protocol for enrichment of native O-GlcNAcylated peptides based on use of the lectin, wheat germ agglutinin (WGA). In addition we have carried out a comprehensive analysis of N-and O-linked glycosylation enriched by our WGA strategy in addition to O-GlcNAc modified peptides. For protein O-GlcNAc per se, we have focused efforts on deciphering the site specific functions of O-GlcNAcylation in several biological settings including the synapse, regulation of the circadian clock and stem cell pluripotency. Studies of the murine synapse have explored a sequential enrichment strategy for both O-GlcNAcylation and phosphorylation from the same biological preparation. This work has revealed some 1600 sites of O-GlcNAcylation and over 16,000 sites of phosphorylation. In addition, we have shown dynamic involvement of O-GlcNAcylation with phosphorylation of a region of PER2 known to regulate human sleep phase. Finally we have established the role of sox2 O-GlcNAcylation in the efficiency of somatic cell reprogramming to iPSCs. This presentation will outline our current understanding of O-GlcNAc function and its relation to S/T phosphorylation. Financial support was provided by the NIH NIGMS Grant P41GM103481, the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, and the Howard Hughes Medical Institute (to ALB).

(3) Glycomics and glycoproteomics-providing new biological insights

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Glycans, in the form of polysaccharides or glycoconjugates, are the most abundant class of biomolecules. Every cell has multiple types of glycan on its surface where they play vital roles in cell-cell and cell-matrix interactions. Structural determination is essential for understanding the roles that glycans play in biological systems. Mass spectrometry (MS), with its ultra-high sensitivity and ability to analyse complex mixtures of glycans, is the most powerful tool currently available for glycan structure analysis. Our laboratory is engaged in numerous world-wide collaborations in which we exploit high sensitivity mass spectrometric methodologies for the structural characterisation of glycans found in a diverse range of biological material. This presentation will review MS strategies incorporating MALDI-TOF-TOF MS/ MS and nanoLC-ES-MS/ MS for defining the glycomes of cells, tissues and purified glycoconjugates as well as establishing glycoprotein site-specific glycosylation. The broad range of our research activities will be illustrated by data from ongoing collaborative projects embracing host pathogen interactions and human disease.

(4) Role of O-glucose glycans in animal development

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One of the post-translational modifications occurring on epidermal growth factor-like (EGF) repeats is the addition of an O-linked glucose to the serine residue in the C-X-S-X-P/A)-C consensus sequence, which is present in a relatively small number of EGF repeats. Genetic, cell culture and biochemical experiments indicate that an evolutionarily conserved enzyme called Rumi (Poglul1) is
responsible for protein O-glucosylation in *Drosophila* and in mammals. Loss of Rum in *Drosophila* results in a temperature-sensitive loss of Notch signaling. Moreover, mutating the Rum target sites in a Notch genomic transgene recapitulates the rumi loss-of-function phenotypes in flies, indicating that the Notch receptor is a biologically-relevant target of Rum. Although Notch receptors have by far the largest number of Rum target sites among animal proteins, a number of other proteins harbor EGF repeats with Rum target sites as well. We therefore asked whether Rum is required for the function of proteins other than the Notch receptor, and identified a phenotype in *rumi* mutant flies that cannot be explained by loss of O-glucose from Notch. I will present our recent data on the identification of a new target of Rum and on the potential mechanism through which loss of O-glucose from this target results in a developmental phenotype in *rumi* mutants. This work was supported by the Mizutani Foundation for Glycoscience (H.J.-N.) and the NIH (R01GM084135 to H.J.-N., R01GM061126 to R.S.H.) A.R.H. and T.V.L. contributed equally to this work.

(5) The in vivo function of *Drosophila* CMP-Sialic acid synthetase

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Sialylation is ubiquitous in vertebrates playing a vital role in many biological processes including immune responses, cell adhesion and signaling events, and neural development. Sialylation has been extensively studied in mammals, yet its genetic and molecular mechanisms are not well understood given the complexity of mammalian glycosylation pathways. We utilize *Drosophila* as a model with simplified sialylation and well-characterized neural development. Our lab has previously described the function of DSiaT, the sole *Drosophila* sialyltransferase closely related to ST6Gal family of mammalian sialyltransferases. DSiaT was found to modulate neural transmission and development. In order to better understand the regulation of sialylation in neurons, we continued our study of the biosynthesis of sialylated glycoproteins in *Drosophila* by investigating the function of CMP-Sialic acid synthetase (CSAS). CSAS is the enzyme producing the donor sugar substrate for DSiaT. CMP-Sia. CSAS is expressed in the CNS throughout development suggesting a nervous system-specific function. Unlike mammalian CMP-Sia synthetases which are found in the nucleus, *Drosophila* CSAS is present in the Golgi and colocalizes with DSiaT. By employing genetic and behavioral assays we found that CSAS mutants generally mimic DSiaT mutations, confirming its role in the sialylation pathway. Similar to DSiaT, CSAS interacts with ion channel genes that control neural excitability. Our electrophysiological assays revealed that CSAS mutants display lowered excitability as shown by a decrease in evoked excitatory neuromuscular junction potential (EJP). Our data indicate that there is a specific pathway that regulates neural transmission and relies on sialylation.

Interestingly, overexpression of CSAS potentiates excitability in neurons, suggesting its activity is necessary and sufficient for establishing the proper level of excitability and indicating a possible regulatory role in the pathway. However, our studies revealed complex genetic interactions between DSiaT and CSAS, suggesting that these genes may have independent functions within the nervous system. These additional functions are possibly separate from their role in generating sialoglycoconjugates, and they may be developmentally and spatially regulated. This work represents the first systematic study of a eukaryotic CMP-Sia synthetase function in vivo. Our findings shed additional light on potentially conserved mechanisms of sialylation and sialylation-mediated control of neural transmission. This project was supported by NIH/NS075534 to VMP.

**Reference**


(6) O-Fucosylation and Development

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O-Fucose is known to be added to distinct consensus sequences in Epidermal Growth Factor-like (EGF) repeats and Thrombospondin Type 1 Repeats (TSRs) by Protein O-fucosyltransferase 1 (Pofut1) and 2 (Pofut2), respectively. Elimination of Pofut1 in mice results in embryonic lethality with severe Notch-like defects supporting an essential role for O-fucosylation of EGF repeats in Notch function. Elimination of Pofut2 in mice also results in a severe embryonic lethal phenotype, although the molecular mechanism for this lethality is unknown. O-Fucose on TSRs is typically elongated to the di-saccharide Glcβ1-3Fuc by a β3-GlcT, mutations in which result in the human genetic disorder Peter’s Plus Syndrome, a severe developmental disorder with multiple effects. Again, the molecular mechanisms for the defects in Peter’s Plus Syndrome are unknown. To better understand the biological role of O-fucose glycans on TSRs, we sought to identify Pofut2 targets by mapping sites of O-fucosylation to predicted sites. Mass spectral glycoproteomic analysis shows that the sites are typically modified at high stoichiometry with the Glcβ1-3Fuc disaccharide. To further define the consensus sequence, we have mutated all residues within the consensus sequence of a model TSR from human thrombospondin 1 and evaluated the efficiency of fucosylation in cells. These results led to a revised consensus sequence, C-X₁-X₂-(T/ S)-C, where T is preferred over S as the modified residue and the presence of D or L in the X₁ position severely reduces fucosylation. This consensus sequence predicts O-fucosylation of 205 TSRs within 52 proteins in mouse or human genomes. Cell-based studies reveal that elimination of O-fucosylation by knocking down Pofut2 with RNAi, reducing GDP-fucose levels in Lec13-CHO cells, or mutating sites of modification, results in secretion defects for TSR-containing proteins. Pofut2 is localized to the ER and O-fucose is added to proteins both co-and post-translationally. O-Fucosylation appears to increase as TSRs fold in the ER, serving as a marker for folding. *In vitro* assays

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with an individual TSR show that Pofut2 accelerates the rate of TSR folding, strongly suggesting that Pofut2 is a chaperone for TSRs. This work was supported by NIH grant CA12307101.

(7) Glycosyltransferases as systemic regulators of blood cell development
Joseph Lau
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Glycosyltransferases, which normally reside within the intracellular secretory apparatus to assemble glycans on nascent proteins and lipids in transit, are also present in abundance in the extracellular milieu, especially in systemic circulation. Blood-borne enzymes include glycosyltransferases that can assemble β3- and β4-galactosides, α2-, α3-, and α4-fucosides, as well as α3- and α6-sialylsides to generate a plethora of physiologically important structures including Lewis and sialyl Lewis structures. Little is known of the physiologic values of the extracellular glycosyltransferases in circulation. We show that the blood-borne sialyltransferase, ST6Gal-1, attenuates hematopoietic extracellular glycosyltransferases in circulation. We show that the blood-borne sialyltransferase, ST6Gal-1, attenuates hematopoietic stem and progenitor cell (HSPC) proliferation and differentiation. In bone marrow chimeras, α6-sialylation of HSPCs is profoundly dependent on the levels of circulatory ST6Gal-1 of the recipients, and is independent of the HSPC’s inherent ability to express endogenous ST6Gal-1. In fact, murine HSPCs operationally defined as Lin-c-Kit + do not express endogenous ST6Gal-1 but their cell surfaces are amply decorated with α6-sialyl to GlcNAc structures. We propose that remotely produced, rather than endogenously expressed enzyme, is the principal modifier of HSPCs glycans for α6-sialic acids. In so doing, liver-produced ST6Gal-1 may be a potent systemic regulator of hematopoiesis. (Fundied by NIH Program of Excellence in Glycosciences P01HL107146 and NIH R01AI56082)

(8) Possible Role of Cathepsin-Mediated Growth Factor Activation in the Pathogenesis of Mucolipidosis II
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The lysosomal disorder mucolipidosis II (ML-II) is caused by defective biosynthesis of the mannos 6-phosphate tag responsible for the targeting of acid hydrolases to the lysosome. Upon loss of this tag, the hydrolases are hypersecreted from the cell. The mechanisms whereby this lysosomal mistargeting results in the abnormal skeletal and cartilage development associated with ML-II have not been fully elucidated. Using a zebrafish model for ML-II, we previously demonstrated abnormal cartilage development characterized by the sustained expression of type II collagen, a transforming growth factor beta (TGFbeta)-regulated ECM protein. Increased and sustained activity of cathepsin K (ctsK) was noted in ML-II embryos, and suppression of this activity reduced type II collagen staining and normalized chondrocyte development. This finding suggests an unexpected relationship between excessive cathepsin activity and sustained collagen expression. Our current evidence indicates that altered chondrogenesis in ML-II zebrafish embryos correlates with an imbalance in the growth factor signaling pathways that drive chondrocyte development. In an effort to link cathepsin activity with this imbalance, we used in vitro digestion assays to test the possibility that cathepsin proteases are not active on latent growth factors, resulting in their promiscuous activation. Our results show that the latency-associated peptide (LAP) of TGFbeta, responsible for maintaining this growth factor in an inactive state, is a substrate for cathepsins D and K at acidic pH. Importantly, LAP can also be efficiently degraded by ctsK at neutral pH, consistent with its ability to activate this growth factor within the extracellular space that surrounds chondrocytes. Under these conditions, the mature TGFbeta ligand was stable, indicating that latent TGFbeta can be effectively activated by cathepsin activity. Analysis of cathepsin specificity towards latent TGFbeta binding proteins and other growth factors is ongoing. Collectively, our results uncover a previously unrecognized mechanistic link between extracellular cathepsin activity and the altered growth factor signaling that may underlie abnormal chondrogenesis in ML-II zebrafish.

(9) A murine knockout of the human diabetes susceptibility locus Oga contributes to acute and chronic nutrient sensing defects
Dona Love, Chithra Kembiyethetty, Oksana Gavrilova, Marcy Comly, Katryn Harwood, John Hanover
NIDDK, National Institutes of Health

Nutrient flux within the intracellular environment contributes to both acute and chronic complications for the offspring. For example, children of diabetic mothers have a higher incidence of birth defects and an increased occurrence of obesity and diabetes as an adult. O-GlcNAc cycling is an essential nutrient-sensing pathway linking nutrient flux to cellular signaling, transcription and chromatin remodeling and may become deregulated in the diabetic state. The removing enzyme O-GlcNAcase (OGA) is a diabetes susceptibility locus and together with the O-GlcNAc transferase (OGT) maintains the dynamic cycling of this simple sugar at serine and threonine residues. Since deletion of X-linked, Ogt in mammals is lethal, we deleted the removing enzyme, Oga, to examine impact of excess O-GlcNAc on nutrient sensing and development. The most acute defect, contributing to a 3% survival rate, appeared to be a failure in nutrient homeostasis. Neonatal null pups had significantly lower hepatic glycogen and circulating glucose than their wild-type littersmates. Chronic nutrient sensing defects, consistent with insulin resistance, were detected in the surviving Oga null animals and their heterozygous littermates. Additionally, these null mice exhibited homeotic transformations and hematopoietic stem cell defects, phenotypes associated with aberrant Polycomb Group repression. High-throughput ChIP-seq and gene expression analysis revealed promoters marked by O-GlcNAc, including many of the Hox genes and confirmed their deregulation. Taken together, O-GlcNAc cycling represents a mechanism linking nutrient flux to cellular signaling and the epigenetic machinery and suggests a model by which nutritional information may be transmitted across generations.
Cellulose is the most abundant polymer on earth. It is an extracellular linear polymer of glucose molecules synthesized by the membrane-embedded cellulose synthase. Cellulose is primarily produced by vascular plants and algae, but also by some bacteria upon formation of biofilms. While polymer synthesis occurs at the cytoplasmic side of the membrane, the polymer is translocated across the membrane during its synthesis through a channel formed by the cellulose synthase. Hence, cellulose synthase combines the functions of a polymer synthase with a translocase. In Gram-negative bacteria, cellulose is synthesized and translocated across the inner membrane by a complex consisting of the catalytic BcsA and the periplasmic BcsB subunits. BcsA contains eight transmembrane helices, which form a pore for the growing polysaccharide chain. By determining crystal structures of the BcsA-BcsB complex at different stages during cellulose synthesis and translocation, we are able to unravel the mechanism by which cellulose is synthesized and translocated across the cell membrane.

Identification of a GlcA transferase involved in biosynthesis of glycosyl inositol phosphorylceramide sphingolipids in plants

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Glycosyl inositol phosphorylceramide (GIPC) sphingolipids are a major class of lipids in fungi, protozoans, and plants. GIPCs are highly glycosylated and thus have limited solubility in typical lipid extraction solvents, making them difficult to study. However, GIPCs are major lipids in the plant plasma membrane and they are involved in many essential processes, including pathogen defense, symbiosis, and membrane trafficking and organization including formation of lipid rafts. Here we show that one Arabidopsis member of Glycosyltransferase Family 8, IPGT1, is a GlcA transferase involved in GIPC biosynthesis. To determine the function of IPGT1, we used a yeast (Saccharomyces cerevisiae) mutant that accumulates the sphingolipid glycosyl inositol phosphorylceramide (IPC). Since UDP-GlcA is not naturally present in yeast, we engineered the yeast mutant with UDP-Glc dehydrogenase and a human Golgi-localized UDP-GlcA transporter, to make UDP-GlcA available for GlcA transferases. When IPGT1 was expressed in this engineered yeast strain it transferred GlcA to IPC. To further investigate the activity of IPGT1 in planta, we overexpressed or silenced it in Nicotiana benthamiana. Microsomes from plants transiently overexpressing IPGT1 incorporated radiolabel from UDP-[3H]GlcA into sphingolipids, while microsomes from plants in which IPGT1 was silenced incorporated less radiolabel. Mutations in the IPGT1 gene are pollen lethal, indicating that these sphingolipids are essential in plants.

GDP-Mannose transport in Cryptococcus neoformans is mediated by two distinct proteins and is dispensable for viability

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Cryptococcus neoformans is an opportunistic pathogen responsible for cryptococcal meningoencephalitis. Every year this disease kills over 600,000 people who are immunocompromised due to AIDS or other conditions. The fungal pathogen is surrounded by a polysaccharide capsule, which is its major virulence factor. The capsule is composed primarily of two polysaccharides, glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal); mannose comprises over half of the capsule mass. C. neoformans also extensively utilizes mannose in cell wall synthesis and glycosylation of proteins and lipids. GDP-mannose (GDP-Man) is produced in the cytosol by the sequential actions of phosphomannose isomerase, phosphomannomutase, and GDP-Man pyrophosphorylase. However, most of the glycan synthetic reactions for which this compound serves as the donor occur in the Golgi complex. This highly charged compound thus requires specific nucleotide sugar transporters to convey it to the site of these biosynthetic reactions, similar to the case for many other nucleotide sugars. Transport of GDP-Man is of particular interest in the context of a microbial pathogen, however, since mammalian cells lack this capability. We previously identified two GDP-mannose transporters in C. neoformans, which we termed Gmt1 and Gmt2. Biochemical studies of each protein expressed in Saccharomyces cerevisiae showed that both are functional, with similar kinetics and substrate specificities. Surprisingly, microarray experiments indicated that the genes encoding Gmt1 and Gmt2 are transcribed with distinct patterns of expression in response to variations in growth conditions. To investigate potential functional differences between these two transporters, we first generated a double mutant strain. We were surprised that this strain was viable, since the single gene encoding a GDP-Man transporter in S. cerevisiae is essential. We next compared cell growth, colony morphology, protein glycosylation, and capsule phenotypes of the Gmt single and double mutants. In all of these studies, the gmt1 mutant showed significant phenotypic differences from gmt2 mutant, suggesting the two proteins play different roles in cryptococcal biology. This hypothesis was supported by nonidentical subcellular localization of the Gmts, as defined by immunofluorescence microscopy. We also found that the double mutant exhibited severe defects in capsule synthesis and protein glycosylation, and was completely avirulent in mouse models of cryptococcal infection.
Leishmania parasites elaborate a sophisticated glycoalyx playing key roles in the infectious cycle. The major glycoconjugate is lipopolysaccharide (LPS), which is comprised of a GPI anchored polymer of substituted [Galβ(1,4)-Man(α1)-PO] repeating units. The phosphoglycan repeats (PGs) are also expressed on the less abundant proteoglycans (PGG) and secretory acid phosphatase (SAP). The structural diversity and biosynthetic compartmentalization of these glycoconjugates suggest the likelihood of multiple enzymes for their assembly. Focusing on the key Man-P trans-ferases, previous studies suggested the presence of ‘initiating’ MPT (eMPT) using the LPG GPI-core as acceptor, and a separate "elongation" MPT (eMPT) using subsequent PG units as acceptors. Similarly PPGs require iMPT and eMPTs, although the PPG iMPT must add Man-P to Ser residues of the PPG protein backbone. We have used functional rescue studies to identify a gene encoding the eMPT, belonging to 3 genes of the LPG4 family. We generated gene knockouts of each gene in Leishmania major and assayed the conse-quences of glycoconjugate synthesis. Ablation of LPG4B and LPG4C resulted in complete loss of LPG, and biochemical characteriza-tion and enzymatic assays of lpg4b and lpg4c mutants established that LPG4B encodes the LPG iMPT and LPG4C the LPG eMPT. PPG synthesis was largely unaffected in these mutants. In contrast, lpg4a mutants showed no effect on LPG synthesis (pro-cyclic and the longer metacyclic form of LPG) but showed decreased PG modification of PPGs and several transfected PG-modified protein reporters (PPG1, SAP). These data suggest that LPG4A encodes the PPG eMPT activity, leaving the PPG iMPT to be identified in ongoing work. Notably, the predicted LPG4 proteins bear several motifs shared by bacterial capsular genes implicated in hexose-P transfer, raising the possibility that the LPG4 genes were acquired by horizontal gene transfer during evolution.

(14) Signaling By Free Mannose-6-Phosphate

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Our laboratory studies free mannose-6-phosphate (M6P) as a second-messenger signaling molecule, aside from its well-known role in glycoconjugate metabolism and independently of the M6P receptor system. Signaling requires the unique ability of M6P, unlike other sugar phosphates, to trigger hydrolysis of the mature LLO “G M Gn-P-P-Dolichol” which is an essential glycan donor for N-linked glycosylation (JBC 250:17901). Premature LLOs remain unaffected. Consequently, mature LLO is depleted and free G M Gn is delivered into the ER lumen. This reaction explains why accumulation of M6P, and not depletion of MIP, best accounts for LLO loss in a zebrafish model of the disease PMM2-CDG in which the M6P to MIP converting enzyme phosphomannomutase is deficient (MBoC 23:4175). M6P second-messenger activity is the terminus of a novel signaling pathway that responds to endoplasmic reticulum stress, including the stress of infection by the enveloped virus HSV1 which is coated with glycoproteins. In this pathway, activation of the kinase IRE1α by stress triggers glycogen breakdown for M6P synthesis, and then LLO cleavage (MBoC 22:2994). The sensor and effector of the M6P second-messenger are unknown, and it is not clear whether its synthesis involves a specialized pathway to insulate M6P signaling from conventional glycogen metabolism. Further, since enveloped viruses require LLO, it remains to be determined if LLO depletion by M6P signaling is a form of innate immunity for suppressing viral in-fection. Our recent efforts to answer these important questions will be presented. Supported by NIH grant GM038545.

(15) Mucin-type O-glycosylation is required for polarized secretion in the Drosophila digestive system

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Polarized secretion is an important cellular process that plays critical roles in development and disease. Here we found that mucin-type O-glycosylation functions as a novel regulator of polarized secretion in specialized secretory cells of the Drosophila digestive system. Certain members of the large enzyme family responsible for the initiation of mucin-type O-glycosylation are expressed specifically in a subset of Drosophila foregut cells (PR cells) that are responsible for secreting the peritrophic membrane of the digestive system. Mutations in or RNAi to certain members resulted in lethality and defective proventriculus for-mation, with larger and minimally-shaped PR cells. Furthermore, we observed disrupted apical secretion of ECM proteins, abnormal secretory granule formation, and altered localization and structure of the secretory apparatus in PR cells. We have identified protein targets of these enzymes. We propose that O-glycosylation is responsible for sta-bilizing certain proteins that play important roles in polarized secretion within the digestive system. This study demonstrates that mucin-type O-glycosylation is required for polarized secretion in vivo and provides insight into the relationship between abnormal O-glycosylation and diseases of the digestive tract that are seen in mammals.

(16) Characterizing a missense mutation in O-GlcNAc transferase that segregates with disease in a family with X-linked Intellectual Disability

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Beta-N-acetylglucosamine (O-GlcNAc) is a dynamic post-translational modification that is covalently attached to serines and threonines of nuclear and cytoplasmic proteins, and has been recently implicated in epigenetic regulation. Single genes encode enzymes for its attachment [O-GlcNAc transferase (OGT)] and removal [O-GlcNAcase (OGA)]. An X-chromosome exome screen identified a missense mutation in the tetramericopeptide repeat (TPR) region [762G>T (p.L254F)] of OGT that segregates with X-linked
intellectual disability (XLID) in a family. Patients exhibited hypopadria, clinodactyly, short stature, microcephaly, and ID. A decrease in steady-state OGT protein levels was seen in isolated lymphoblastoid cell lines from two patient samples, harboring L254F-OGT, compared to a carrier. Surprisingly, steady-state global O-GlcNAc levels remained grossly unaffected. The same samples, however, showed a decrease in steady-state OGA levels. These findings imply a compensation mechanism exists, though imperfect given the phenotype of the patients, for maintaining global O-GlcNAc levels. L254F-OGT patients also show a decrease in OGA mRNA and luciferase reporter expression leading us to hypothesize that OGT regulates the transcription of OGA in a mechanism to be elucidated. Currently, we are examining the stability, activity, and half-life of the OGT mutant protein compared to wildtype, examining OGT-mediated regulation of OGA transcription as well as global gene expression, and examining the impact of the mutant on the cellular O-GlcNAcome. Finally, studies are underway to generate induced pluripotent stem (iPS) cells from affected patients and unaffected relatives in order to explore the imperfect compensatory mechanism in cell type specific contexts with a focus on neural lineages due to the specific phenotypes observed.

(17) SGK196 Is a Glycosylation-Specific O-Mannose Kinase Required for Dystroglycan Function
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Dystroglycan is a critical component of the dystrophin-glycoprotein complex, and it binds to extracellular matrix (ECM) ligands including laminin, agrin, and perlecain at the cell surface. The malfunction of dystroglycan as an ECM receptor are a common to a variety of congenital muscular dysstrophies (CMDs)-including Walker-Warburg syndrome, Fukuyama CMD, Muscle-Eye-Brain disease, and certain types of limb-girdle muscular dystrophy. These clinical findings underscore the fact that a tight association between the basement membrane and sarcosome through dystroglycan is fundamental to maintain muscle integrity. Dystroglycan’s ability to serve as an ECM receptor requires various types of post translational modification. In particular, phosphorylation at the 6-position of an O-mannosyl trisaccharide [N-acetylgalactosamine (GalNAc)-β3-N-acetylgalactosamine (GlcNAc)-β4-mannose] produces a branch chain that is ultimately extended with repeating disaccharides [-α3-glucuronic acid (GlcA)-β3-xylose (Xyl)-] synthesized by like-acetylgalactosaminyltransferase (LARGE), enabling dystroglycan to bind the ECM ligands. However, the synthetic pathway that produces this structure is largely unknown. Here, we found that GTDC2 is localized at the ER and has a protein O-mannose β1,4-N-acetylgalactosaminyltransferase activity, which leads us to designate it as POMGNT2. We also demonstrated that GTDC2 and B3GALNT2 can synthesize a GalNAc-β3-GlcNAc-β-terminus at the 4-position of protein O-mannose. Newly identified CMD causative protein: SGK196 phosphorylates the 6-position of O-mannose using ATP, based on which we propose to designate it as a protein O-mannose kinase (POMK). Because SGK196 lacks certain catalytic residues conserved among protein kinases, its manner to catalyze the phosphotransfer reaction may be unlike any other protein kinase known to date. SGK196 exhibits the phosphorylation activity only when the GalNAc-β3-GlcNAc-β-terminus is linked to the 4-position of O-mannose, indicating that this disaccharide serves as the substrate recognition motif of SGK196. This strict specificity of SGK196 explains why mutations in GTDC2 and B3GALNT2 cause congenital muscular dystrophy although their product does not directly recognize the ECM ligand.

(18) Differential Golgi targeting of βgalactosidase:α2-3sialyltransferase-1 and core 2 N-acetylgalcosaminyltransferase-1/1 in advanced prostate cancer cells
Armen Petrosyan, Pi-Wan Cheng
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We recently have shown glycosyltransferase-specific Golgi targeting mechanisms: Core 2 N-acetylgalcosaminyltransferase-M/2 (C2GnT-M/2), which contains N-glycans, utilizes Giantin while core 1 β3 galactosyltransferase 1 (C1GalT1), which is free of N-glycans, employs GM130-GRASP65 or GM130-Giantin in the absence of GRASP65 (JBC 287:37621, 2012). Here, we found that C2GnT-L/1, which is localized to the Golgi of androgen-sensitive LNCaP cells, is in the endoplasmic reticulum (ER) of androgen-refractory PC-3 and DU145 cells while C1GalT1 and βgalactosidase:α2-3sialyltransferase-1 (ST3Gal1) are in the Golgi of all three cells. We were puzzled by the differential Golgi and ER distribution of ST3Gal1 and C2GnT-L in these aggressive prostate cancer cells because like C2GnT-L, ST3Gal1 also contains N-glycans and was predicted to use same Golgi targeting mechanism. We also found that contrary to LNCaP, both DU145 and PC-3 cells have fragmented Golgi and lack Giantin. Giantin was the main golgin responsible for reformation of Golgi morphology in LNCaP cells after treatment with Golgi disruptive agent, Brefeldin A. C1GalT1 and ST3Gal1 were instantly detected in the re-formed Golgi stacks while movement of C2GnT-L from ER to Golgi did not occur until after the Golgi morphology was completely restored. Without Giantin, Golgi morphology could not be restored and C2GnT-L was retained in the ER, however both C1GalT1 and ST3Gal1 were detected in the reformed Golgi. The results suggest that C1GalT1 and ST3Gal1 can target to the fragmented Golgi utilizing Giantin-independent mechanism. Indeed, knockdown of GRASP65 or GM130 in DU145 and PC-3 cells results in retention of these enzymes in the ER. We hypothesized that the Giantin in PC-3 and DU145 cells was altered as a result of downregulation of its chaperone, protein disulfide isomerase A3 (Mol Cancer 8:130, 2009). Thus, in androgen-refractory cells, C2GnT-L was not able to reach the Golgi due to defective Giantin and retained in the ER to be degraded. This property coupled with the capability of ST3Gal1 to...
reach the Golgi using the GM130-GRASP65 complex could explain the reported O-glycosylation dominated by ST3GAlI instead of C2GnT-L in advanced prostate cancer cells (Cancer Res 67:6155, 2007). (The work is supported by VA Merit Award 1I1BX000985 and Nebraska LB506 grant).

(19) Pathways and regulation of IgA1 O-glycosylation in an autoimmune disease, IgA nephropathy
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IgA nephropathy (IgAN), the most common glomerulonephritis worldwide, is an autoimmune disease wherein immune complexes consisting of IgA1 with galactose-deficient O-glycans (Gd-IgA1; autoantigen) and anti-glycan autoantibodies deposit in the glomeruli and induce renal injury. Defining the nature of the Gd-IgA1 autoantigen in IgAN requires analysis of the clustered hinge-region O-glycans in the context of adjacent sites as well as the heterogeneity at each individual site. Using a combination of approaches and a new glycoproteomic workflow, we are defining the heterogeneity of IgA1 O-glycosylation at the molecular level across experimental and clinical samples. The IgA1 hinge region has 3 to 6 O-glycans and the sites of galactose deficiency may occur at positions Ser230, Thr233, and Thr236 in different combinations, whereas sites IgA1 at positions Thr225, Thr228, Ser232 may be without glycans or with galactosylated O-glycans. IgA1 secreted by IgA1-producing cell lines from IgAN patients has more O-glycans and more galactose-deficient sites compared to IgA1 from cells of healthy controls. The observed changes in patterns of O-glycans of IgA1 secreted by cells from IgAN patients vs. controls were associated with changes in expression/activity of several key glycosyltransferases. Specifically, we found elevated expression of one of the initiating enzymes, GalNAc-transferase 14 (GalNAc-T14), and elevated expression and activity of GalNAc-specific sialyltransferase (ST6GalNAc-II) and, conversely, decreased expression and activity of the galactosyltransferase (C1GalT1) and decreased expression of the C1GalT1-associated chaperone Cosmc. These findings were confirmed by siRNA knock-down of the corresponding genes (GALNT14, ST6GALNAC2, C1GALT1) and by in vitro enzyme reactions. Expression and/or subcellular localization of some of these enzymes can be regulated by several cytokines that further enhance the imbalance of the glycosyltransferases and, subsequently, enhance galactose deficiency of the IgA1 O-glycans. Genetically determined factors may influence the activity of the glycosyltransferases, as our prior GWAS data indicated several loci associated with Gd-IgA1 production. In summary, Gd-IgA1, the key autoantigen in IgAN, is the result of the dysregulation of multiple enzymes in IgA1-producing cells. These findings thus provide insight into possible targets for future disease-specific therapy.

(20) GOLPH3 regulates integrin-mediated biological functions via up-regulation of sialylation
Tomoya Isaji, Sanghun Im, Tomohiko Fukuda, Jianguo Gu
Tohoku Pharmaceutical University

Recently, a Golgi protein, GOLPH3 was identified as a new onco-gene that is commonly amplified in human cancers. An ortholog of GOLPH3, VPS74p has been reported to be essential for glycosyltransferase activation of yeast. To investigate whether the expression of GOLPH3 was involved in the cell migration and N-glycosylation in mammalian cells, we performed a loss-of-functional study. Cell migration on fibronectin or laminin was suppressed in GOLPH3 knockdown (KD) cells, and the suppression was restored by reintroduction of GOLPH3 gene. The cell migration was abolished in the presence of neutralized anti-beta1 integrin antibody. Interestingly, N-glycosylation status of beta integrin obtained from KD cells was apparently different from those in control cells, while it was normalized in the restored cells. HPLC and LC/MS analysis showed that the sialylation level of N-glycans was specifically decreased in KD cells, and the aberrant N-glycosylation was significantly decreased in the restored cells. To explore the specific effect on sialylation by GOLPH3 expression, we examined the interactions between a glycosyltransferase and GOLPH3, and found GOLPH3 specifically associated with alpha2,6-sialyltransferase I or alpha2,3-sialyltransferase IV, but not beta1,4-galactosyltransferase I. The cytoplasmic tails of sialyltransferases were shown to be important for the association. Interestingly, overexpression of alpha2,6-sialyltransferase I greatly rescued the cell migration and intracellular signaling, which were down-regulated in GOLPH3 KD cells. Taken together, these results suggest that GOLPH3 regulates N-glycosylation status of beta integrin and its biological functions, which may give a new insight for the functions of GOLPH3 in cancer.

(21) Non-lyosomal degradation pathway for N-glycans; cases in yeast and mammalian cells
Tadashi Suzuki
Glycometabolome Team, RIKEN Global Research Cluster

It has been well described that glycans on glycoconjugates are catalyzed in the lysosomes. However, discovery of the cytoplasmic peptide/N-glycanase (PNGase) (Suzuki et al. 1993) led to characterization of another degradation pathway for N-glycans. In mammalian cells, at least 3 enzymes are known to be involved in this novel pathway now called "non-lyosomal" degradation pathway (Suzuki 2001; PNGase, endo-β-N-acetylglucosaminidase (ENGase) (Suzuki 2002), and α-mannosidase (Man2C1) (Suzuki et al. 2006). On the other hand, not much is known about the catabolic pathway for N-glycans in budding yeast (S. cerevisiae), as no apparent homologues, except for the cytoplasmic PNGase, were found for mammalian lysosomal enzymes in yeast. While we analyze the cytosolic free oligosaccharides (fOSs) in yeast, it was found that most, if not all, of fOSs were found to be generated in budding yeast (Hirayama et al. 2010). These results are in sharp contrast to the case in mammalian cells, as most of the fOSs appears to be formed in a PNGase-independent fashion (Chanret et al. 2010). These results...
most likely indicate that the catabolic pathway seems to be quite distinct between human and budding yeast. In this lecture I will highlight the distinct catabolic pathway for N-glycans between mammalian cells and yeast. I will also present our most up-to-date data on the mechanism of PNGase-independent fOSs generation in mammalian cells.

References

(22) Structural insights into novel deoxysugar biosynthesis in bacteria
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University of Wisconsin-Madison

Recent years have witnessed an explosion in research efforts directed at understanding the biosynthesis of unusual deoxysugars. These carbohydrates are produced by a variety of bacteria, fungi, and plants, and are found, for example, on antibacterial, antitumor, and/or antifungal agents. One such sugar is L-epivancosamine, a 2,3,6-trideoxy sugar observed on the aglycone scaffold of the antibiotic chloroeremomycin. Interestingly, chloroeremomycin has been shown to be a more potent antibiotic than vancomycin, presumably due to the presence of L-epivancosamine. Indeed, the di- and tri-deoxysugars are often critical for the efficacies of the compounds to which they are attached. A key step in the biosynthesis of L-epivancosamine is the removal of the hexose C-2' hydroxyl group and the oxidation of the C-3' hydroxyl group to a carbonyl moiety. The enzyme responsible for this is referred to as EvaA, a 2,3-dehydratase. The 2,3-dehydratases have been, for the most part, largely uncharacterized. The focus of this presentation is on the structure and function of EvaA. The structure of the enzyme was solved by X-ray crystallography to a nominal resolution of 1.7 Å. Each subunit of the dimeric protein folds into two domains, which are related by a twofold rotational axis and which clearly arose via gene duplication. Strikingly, two binding sites for the dTDP-sugar ligands, referred to as Pockets A and B, have been identified in each subunit. Site-directed mutagenesis experiments and activity assays strongly suggest that Pocket A represents the active site, and Pocket B is a vestige due to the gene duplication event. The overall molecular architecture of EvaA places it into the well-characterized “Nudix” hydrolase family, members of which often function as “housekeeping” enzymes. EvaA is a highly unusual member of this superfamily, however, in that it does not contain the characteristic signature sequence motif, does not bind or require Mg++ for activity, and does not catalyze a hydrolysis reaction but rather a 2,3-dehydration. Taken together, the results from this investigation provide a molecular foundation for understanding the sugar 2,3-dehydratases, whose structures have remained elusive until now.

(23) Discovery of novel toxin binding partners using photocrosslinking sialic acid
Amberlyn M. Wands, Akiko Fujita, Janet E. McCombs, Jennifer J. Kohler
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Many bacterial toxins exploit host glycoconjugates in order to bind to and enter host cells. In the case of cholera toxin, the ganglioside GM1 is the recognized receptor. While cholera toxin subunit B binds GM1 with high affinity, this ganglioside is found at extremely low abundance in the human intestinal epithelium. The low level of GM1 found in host cells raises the possibility that molecules other than GM1 function in cholera toxin binding. Using a metabolically incorporated, photocrosslinking sialic acid analog, we show that cholera toxin subunit B binds and crosslinks to O-linked glycoproteins displayed on the surface of intestinal epithelial cell lines. Mass spectrometry analysis of the crosslinked complex identified candidate cholera toxin-binding glycoproteins. Functional assays show that O-linked glycoproteins are required for cholera toxin-induced cAMP production and chloride ion secretion. These results indicate that O-linked glycoproteins play an essential role in intestinal epithelial cell intoxication by cholera toxin. In addition, the existence of an alternative cholera toxin binding partner provides an explanation for cell type differences in CTx endocytosis.

(24) Further characterization of endogenous mouse lung proteins bearing glycan counter-receptors for Siglec-F
Toshihiko Kato1, Takumi Kiwamoto2, Christopher Evans3, Mary Brummet2, Sherry Hudson2, Zhou Zhu2, Bruce Bochner2, Michael Tiemeyer4
1Complex Carbohydrate Research Center, University of Georgia; 2The Johns Hopkins University School of Medicine; 3Department of Medicine, Division of Pulmonary Medicine, University of Colorado School of Medicine

Lung inflammatory diseases (LIDs) dramatically impact quality of life and affect growing numbers of individuals. Characterized by overly exuberant inflammatory responses, new treatment strategies for LIDs might reasonably target these responses, such as the eosinophilia associated with asthma. Mouse Siglec-F, a functional paralog of human Siglec-8, is a pro-apoptotic receptor expressed by eosinophils associated with asthma. Mouse Siglec-F, a functional paralog of human Siglec-8, is a pro-apoptotic receptor expressed by eosinophils that specifically recognizes sialylated, sulfated glycans on glycan microarrays. However, endogenous tissue sialoside ligands remain unknown. We have previously reported the identification of mouse MUC5B, harvested from mouse tracheal epithelial cells (mTEC), and mouse lung tissue homogenates, as an endogen-ous protein that bears Siglec-F ligands. Here, we further characterize Siglec-F binding glycoproteins. Histochemistry of lung airway tissue sections with Siglec-F-Fc detected the presence of ligand in eosinophils associated with asthma. Many bacterial toxins exploit host glycoconjugates in order to bind to and enter host cells. In the case of cholera toxin, the ganglioside GM1 is the recognized receptor. While cholera toxin subunit B binds GM1 with high affinity, this ganglioside is found at extremely low abundance in the human intestinal epithelium. The low level of GM1 found in host cells raises the possibility that molecules other than GM1 function in cholera toxin binding. Using a metabolically incorporated, photocrosslinking sialic acid analog, we show that cholera toxin subunit B binds and crosslinks to O-linked glycoproteins displayed on the surface of intestinal epithelial cell lines. Mass spectrometry analysis of the crosslinked complex identified candidate cholera toxin-binding glycoproteins. Functional assays show that O-linked glycoproteins are required for cholera toxin-induced cAMP production and chloride ion secretion. These results indicate that O-linked glycoproteins play an essential role in intestinal epithelial cell intoxication by cholera toxin. In addition, the existence of an alternative cholera toxin binding partner provides an explanation for cell type differences in CTx endocytosis.

Lung inflammatory diseases (LIDs) dramatically impact quality of life and affect growing numbers of individuals. Characterized by overly exuberant inflammatory responses, new treatment strategies for LIDs might reasonably target these responses, such as the eosinophilia associated with asthma. Mouse Siglec-F, a functional paralog of human Siglec-8, is a pro-apoptotic receptor expressed by eosinophils that specifically recognizes sialylated, sulfated glycans on glycan microarrays. However, endogenous tissue sialoside ligands remain unknown. We have previously reported the identification of mouse MUC5B, harvested from mouse tracheal epithelial cells (mTEC), and mouse lung tissue homogenates, as an endogenous protein that bears Siglec-F ligands. Here, we further characterize Siglec-F binding glycoproteins. Histochemistry of lung airway tissue sections with Siglec-F-Fc detected the presence of ligand in the airway epithelium and tracheal submucosal glands in wild type and MUC5AC-deficient mice, while MUC5B-deficient mice had diminished levels in the glands but not in the epithelium. Further characterization of the O-linked glycan profile of purified airway mucins by in-gel beta-elimination and nanospray ionization mass spectrometry revealed a range of Core 1, Core 2 and Core 3
O-linked glycans with terminal sialic acid residues. Unexpectedly, based on previous glycan array binding studies, none of the detected O-glycans carried sulfate in the 6'-position of the sialyl-LacNAc motif. Removal of sulfate from mucin preparations by solvolysis did not eliminate Siglec-F-Fc binding, consistent with the observation that Siglec-F ligands are expressed in K562Ga6ST/ C6ST-1 double knock-out mice (Pattnode et al. JBC 2013). Purified airway mucins bound to mouse eosinophils and induced their subsequent death. Taken together, these data identify a previously unrecognized endogenous anti-inflammatory property of mucins by which their α2,3 sialylated glycans can control lung eosinophilia via engagement of Siglec-F.

(25) Context dependent glycan recognition through the next generation of glycan microarray
Geert-Jan Boons
University of Georgia

A major obstacle to advances in glycobiology is the lack of pure and structurally well-defined carbohydrates and glycoconjugates. We have developed a chemo-enzymatic methodology that makes it possible to prepare libraries of highly complex asymmetrically substituted glycans. A key feature of the technology is the use of a core pentasaccharide that at key branching positions is modified by the orthogonal protecting groups to allow selective attachment of unique saccharide structures by chemical glycosylation methodology. The appendages were selected in such a way that the antenna of the resulting compounds can be uniquely extended by glycosyl transferases to give large numbers of highly asymmetrical substituted multi-antennary glycans. The power of the methodology was demonstrated by the preparation of a tri-antennary oligosaccharide that can inhibit binding of spermatozoan to the zona pellucida of human oocytes. Furthermore, we have prepared a series of complex oligosaccharides that were printed as microarrays and screened for binding to lectins and influenza-virus hemagglutinins, which demonstrated that recognition is modulated by presentation of minimal epitopes in the context of complex N-glycans

(26) Reductionist model systems to probe biological and molecular mechanisms of O-GlcNAc signalling
Daniel van Aalten
University of Dundee

MRC Protein Phosphorylation and Ubiquitylation and Division of Molecular Microbiology, College of Life Sciences, University of Dundee, United Kingdom The transient O-GlcNAc modification of nucleocytoplasmic proteins in eukaryotes has been implicated as a signalling modifier of many cellular processes including transcription, stress response, glucose homeostasis, neuronal development, innate immunity and apoptosis. Over 1000 different O-GlcNAc modified substrates have been identified in the human cell but only few of these have been studied in terms of mechanistic biology of O-GlcNAc modification, hampering efforts to identify unifying principles of O-GlcNAc signalling and its effects on other regulatory posttranslational modifications. Furthermore the mechanisms by which O-GlcNAc transferase and O-GlcNAcase are regulated and recognize/ select their protein substrates are unknown. In an attempt to discover reductionist models to study these basic questions in O-GlcNAc signalling, we have used bioinformatic methods to identify a prokaryote and an early eukaryotic ancestor that have clear functional OGA/ OGT orthologues that are active both in vitro and in vivo, and can be probed with inhibitors developed in my laboratory. These enzymes provide tractable routes towards the study of OGA/ OGT-substrate protein complexes by structural biology. Preliminary mass spectrometric evidence suggests the presence of a simple, inducible, O-GlcNAc proteome that allows a route towards probing the early developing roles of O-GlcNAc signalling and its role in the development of complex multicellular organisms. These data represent the first example of O-GlcNAc signalling in early evolution. I will describe the latest unpublished results of this approach.

(27) O-GlcNAc Regulated Arginine Methylation: A Novel Paradigm in Survival Signaling
Albert Lee, Kamau Fahie, Roger Henry, Devin Miller, Natasha Zachara
Johns Hopkins University School of Medicine

The dynamic modification of intracellular proteins by monosaccharides of O-linked β-N-acetylglucosamine (O-GlcNAc) has emerged as a novel regulator of cytoprotection. In response to cellular stress and injury, O-GlcNAc levels are elevated on numerous proteins in a dose-dependent manner. Suggesting that this is an endogenous survival signal, elevating O-GlcNAc levels before or after the induction of injury is protective in both in vivo and in vitro models. In order to investigate the molecular mechanisms that underlie this protective phenotype, we have identified proteins whose O-GlcNAcylation status changes with cellular injury. In this study, we report that protein arginine methyltransferase 1 (PRMT1) is O-GlcNAc modified and associates with the O-GlcNAc transferase (OGT; catalyzes the addition of O-GlcNAc). Suggesting that OGT/ O-GlcNAc regulates the activity of PRMT1, deletion or inhibition of OGT leads to an increase in the arginine modification catalyzed by PRMT1: asymmetric dimethylation. In vitro assays demonstrated that the physical association of OGT with PRMT1 leads to an inhibition of PRMT1 activity. To assess the role of O-GlcNAc regulated arginine methylation in cellular injury, PRMT1 activity was inhibited pharmacologically or genetically in cells with lower levels of O-GlcNAc. Cells with reduced O-GlcNAcylation are more sensitive to oxidative stress when compared to untreated cells, and this sensitivity can be ablated by PRMT1 knockdown or inhibition. PRMT1 is thought to promote cell death by methylating proteins such as Bad and Foxo, preventing their phosphorylation by Akt. Consistent with a hypothesis in which O-GlcNAc promotes Akt phosphorylation and survival, the pro-apoptotic protein Bad is hypophosphorylated in the OGT null and this can be reversed by inhibition or knockdown of PRMT1. Together, these demonstrate that OGT may be as important as it’s catalytic
activity in promoting cell survival during injury and highlight one mechanism by which OGT/ O-GlcNAc protects cells and tissues.

(28) Increased O-GlcNAcylation of Mitochondrial Proteins Directly Contributes to ROS Production in Diabetes
Junfeng Ma1, Ting Liu1, Brain O’Rourke2, Gerald Hart1
1Johns Hopkins University Department of Biological chemistry; 2Johns Hopkins University Department of Cardiology

O-linked β-D-N-acetylglucosamine addition (O-GlcNAcylation) plays fundamental roles in many important biological processes, including signal transduction, transcriptional control, cell cycle regulation, protein degradation, and stress responses. Abnormally regulated O-GlcNAcylation is involved in numerous chronic diseases, such as diabetes, Alzheimer disease, and heart failure (Hart et al. 2011). Notably, protein O-GlcNAcylation plays crucial roles in diabetic cardiomyopathy, a well-recognized diabetic complication (Ma and Hart 2013). Hyperglycemia-induced overproduction of reactive oxygen species (ROS), which is mainly produced from the mitochondria electron transport chain, is widely regarded to be the major etiology and contributes to the progression of diabetic cardiomyopathy (Brownlee 2001). However, the mechanism of how hyperglycemia causes ROS production is largely unknown. Our recent data has shown that in comparison to controls, diabetic rats have increased O-GlcNAcylation on cardiac mitochondrial respiratory chain proteins. To investigate whether increased O-GlcNAcylation, caused by hyperglycemia, is a direct contributor to ROS overproduction, we used a specific inhibitor (Thiamet-G) of GlcNAcase, the enzyme which removes O-GlcNAc from targeted proteins. After Thiamet-G treatment, mitochondrial O-GlcNAcylation is elevated while the glucose level does not increase. Thiamet-G treated rats show strikingly enhanced cardiac ROS production and altered oxygen consumption rates. These data suggest that increased O-GlcNAcylation of the mitochondrial respiratory chain, due to hyperglycemia, directly contributes to the production of reactive oxygen species (ROS). Our findings reveal novel insight to the etiology and progression of hyperglycemia-induced diabetic cardiomyopathy. Supported by NIH R01CA42486, R01DK61671, N01-HV-00240, P01HL107153 and the Patrick C. Walsh Prostate Cancer Research Fund. Dr. Hart receives a share of royalty received by the university on sales of the CTD 110.6 antibody, which are managed by JHU.

References

(29) From Carbohydrates to Glycomimetics — Is there a General Recipe?
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In the last two decades, a wealth of both physiological and pathophysiological functions related to carbohydrate-lectin interactions have emerged. However, only a fraction of this validated information launched new therapeutic concepts. While the reasons are manifold, two drawbacks seem to be inherently linked to carbohydrate leads: their low affinity (which is often in the micro- or even milli-molar range) and their non-drug-like pharmacokinetic properties (in particular, high polarity limiting the oral bioavailability and the maintenance of therapeutic plasma levels). First, the reasons for the generally observed insufficient pharmacodynamic (PD) properties of carbohydrate-lectin interactions, i.e. low affinity and short residence time, shall be discussed. Emphasis will furthermore be put on the pharmacokinetic (PK) properties of carbohydrate lead structures and a detailed comparison with the characteristics required for drug-likeness. Finally, the structural reasons for the PK/ PD drawbacks will be analyzed and novel optimization strategies -assisted substantially by structure-based design- will be suggested. In the main part, specific examples from the selectin, siglec and bacterial lectin field demonstrate how these PK/ PD gaps can be closed.

(30) Sweet Contacts -Guiding Inhibition of Carbohydrate-Protein Interactions with NMR
Thomas Peters
Institute of Chemistry, University of Lubeck

Specific protein-carbohydrate recognition plays an important role in a huge variety of biological processes. Examples range from viral infections through immune reactions to aberrant decoration of cell walls with glycan chains in cancer. The ability to interfere with and control such processes in order to combat or prevent disease requires a profound understanding of the basis of specific protein-carbohydrate interactions. Although crystallography has delivered valuable structural data on protein-carbohydrate complexes, attempts to use such data for the rational design of inhibitors with therapeutic potential had limited success. One reason for this is that protein-carbohydrate interactions are rather weak, despite their exquisite specificity. On the other hand, NMR offers a number of techniques that are ideally suited to explore weak ligand-protein interactions. Here, it will be explained how such experiments can be beneficial in the search for compounds that inhibit protein-carbohydrate interactions. A human blood group glycosyltransferase and a norovirus capsid will serve as examples where NMR studies led to the discovery of promising inhibitor prototypes.

Reference
The selectins are known as adhesion molecules but more recently have been shown to directly function in cell activation leading to downstream effects such as venous thrombosis. Rivipansel (GMI-1070) is a rationally designed glycomimetic pan-selectin antagonist that is active in models of vaso-occlusive crisis in sickle cell disease (SCD). Here we show that rivipansel strongly inhibits leukocyte activation with IC50’s in the high nanomolar region which translates into significant inhibition in disease models of deep vein thrombosis. Rivipansel dosed at 20mg/kg BID significantly inhibited thrombus formation [90.6% (p < 0.01) at day 2; 83.6% (p < 0.01) at day 6] without the risk of bleeding seen with low molecular weight heparin (Lovenox). Cell adhesion, leukocyte activation, and thrombosis are all functions that occur during the vaso-occlusive event in SCD patients. SCD patients not in crisis have elevated biomarkers for each of these functions. In a phase I clinical trial, rivipansel treatment was tested on these SCD patients for safety, pharmacology, and effects on functional biomarkers. Rivipansel treatment was well tolerated with no serious adverse events. The serum half-life (t 1/2) was 7 to 8 hours and was independent of dose or dosing frequency. Over 90% of the drug was excreted intact in the urine with no major metabolites after both single and multiple dose administrations. The relationship of Cmax and AUC to dose was linear and predictable, increasing proportionally with dose for both single and multiple dose regimens. The effects of rivipansel on the elevated functional biomarkers of cell adhesion (sE-selectin, sP-selectin, sICAM-1), leukocyte activation (MAC-1 LFA-1, PMA) and thrombosis (TF, TAT) were determined by dosing patients at 20mg/kg followed by a second dose 10 hours later at 10mg/kg. Blood samples were taken from patients prior to dosing and at 4, 8, 24, and 48hrs after the initial loading dose. Rivipansel significantly inhibited expression of the above listed functional biomarkers, with some effects seen out to 48hrs past the loading dose. Based on these results combined strong pre-clinical data, a clean safety profile, and good pharmacokinetics, rivipansel was advanced for testing in Phase 2 clinical trials in SCD patients for treatment of vaso-occlusive crisis.

**Glycan Based Inhibitors of Receptors and Enzymes**

**Bernd Meyer**, Katrin Schaefer, Felix Niemeyer, Wei Nien Liao, Moritz Waldmann, Dennis Wilhelm, Miriam Koetzler, Patrizia Leccese  
*Organic Chemistry, University of Hamburg, 20146 Hamburg, Germany*

We have devised new entry inhibitors against influenza and HIV infections that are carrying carbohydrate components. These inhibitors are hybrid glyco-peptido mimetics that have affinities in the high nanomolar range. We report the synthesis, the binding affinities, binding mode and inhibition constants. We have also designed a new scheme for non-ionic inhibitors against glycosyl transferases that cover the donor and the acceptor site of the enzyme. These inhibitors are composed of carbohydrate fragments and aromatic residues. The best donor specific inhibitors have affinities and the hundred micromolar range and are transferase specific. Work is in progress to improve the affinity and cover both the donor and the acceptor site.

**Controlling O-GlcNAc in models systems using small molecule inhibitors**

Scott Yuzwa, Xiaoyang Shan, Nevena Cekic, Lehua Deng, Julia Heinonen, Keith Stubbs, Tracey Gloster, Yanping Zhu, David Vocadlo  
*Simon Fraser University*

The post-translational O-GlcNAc modification of proteins is implicated in diverse cellular processes ranging from epigenetic regulation of gene expression through to cellular signaling. Dysregulation of O-GlcNAc has been proposed to play roles in the etiology of various diseases including, for example, cancer and Alzheimer’s. The physiological and pathophysiological roles of O-GlcNAc have driven interest in methods to manipulate the activity of O-GlcNAc transferase and O-GlcNAcase, the two enzymes that regulate levels of this modification within cells. Here I describe our research directed at generating inhibitors as tools to control cellular O-GlcNAc levels. I will also describe recent results on the use of these inhibitors within model systems as a means to understand the regulation and roles of O-GlcNAc in health and disease with a particular focus on Alzheimer disease.

**Detection of Glycosyltransferase activities with homogenous bioluminescent UDP detection assay**

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Glycosyltransferases (GTs) play a pivotal role in many biological processes, including cell-cell interactions, cell signaling and bacterial cell wall biosynthesis. These enzymes are widely studied and because of their implication in disease states some are becoming potential drug targets. Thus, assays that monitor glycosyltransferase activities are desirable in order to study and understand their mode of regulation, and to search for their selective and potent inhibitors. Monitoring glycosylation reactions was hampered by the lack of robust non-radiometric assays. Traditional assays for GTs are not easily configured for rapid GT activity detection because they rely on detection of radiolabeled substrate which requires product isolation, the use of non-homogenous antibody based assays or mass spectrometry. UDP-sugar is the most used sugar donor for glycosylating enzymes. In a glycosyltransferase reaction, the UDP moiety is
released as a product. Therefore, an assay that detects UDP as universal product of these reactions would be suitable for monitoring most glycosyltransferases activity. We developed a homogenous, bioluminescent UDP detection assay for measuring glycosyltransferase activity. The assay is performed in one step detection that relies on converting simultaneously the UDP product to ATP, then to light in a robust luciferase reaction. The light output is proportional to UDP concentration from low nM to 25 µM. The assay is highly sensitive and robust, two features that are highly desirable and essential for measuring the activity of the majority of GT classes. Therefore, the UDP detection assay allows significant savings of enzyme usage in GT reactions. The assay is simple, and does not require antibodies, nor modified substrates. This assay can be used with GTs that are tagged, native, free or bound to beads. Examples of various applications of this UDP detection assay (UDP-Glo) will be presented, including studies on specificity of transfer of different sugars to different acceptors by diverse GTs. We will show its utility in screening for specific GT (OGT) inhibitors and the study of their mode of action. The development of this UDP detection assay will make it possible to investigate a large number of GTs and will have significant impact on diverse areas of glycobiology research.

(35) The Expanding World of Bacterial Protein O-linked Glycosylation

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Protein glycosylation, once thought of as solely a eukaryotic feature, has been identified in all forms of life. Knowledge concerning bacterial O-glycosylation has significantly grown in the last decade. O-glycosylation was initially regarded as an eccentricity of a few bacterial species, but it is now evident that many bacteria, including important human pathogens, decorate one or multiple proteins with O-glycans. O-glycosylation in bacteria can be classified based on the requirement of an oligosaccharyltransferase (OTase). OTase-dependent glycosylation utilizes an oligosaccharide synthesized on a lipid carrier that is transferred to proteins en bloc by an O-OTase. OTase-independent glycosylation refers to the pathway in which glycosyltransferases sequentially add monosaccharides directly from their nucleotide-activated forms onto the target proteins. Both systems have been demonstrated to have key physiological roles in bacterial lifestyles and pathogenesis. During my talk, I will focus on the structural diversity, the mechanistical aspects, and the physiological role of this increasingly frequent post-translational protein modification in bacteria. Exploiting glycosylation machineries it is now possible to generate glycoconjugates made of different proteins attached to polysaccharides derived from LPS or capsule biosynthesis. These recombinant glycoconjugates can be exploited for vaccines and diagnostics of bacterial infections. Furthermore, O-glycosylation systems are promising targets for antibiotic development. Technological advances in MS and NMR will facilitate the discovery of novel glycosylation systems. Likely, the O-glycosylation pathways we currently know constitute just the tip of the iceberg of a still largely uncharacterized bacterial glycosylation world.

(36) N-Glycan Biosynthesis and Utilization in African Trypanosomes

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African trypanosomes (Trypanosoma brucei), the causative agent of Human African Trypanosomiasis, are parasitic protozoa transmitted by tsetse flies. Besides directly affecting human welfare, trypanosomes are of interest because of their ancient phylogenetic status. Trypanosomes have a fairly typical secretory system that is streamlined for efficient transport of the major virulence factor, glycosylphosphatidylinositol-anchored variant surface glycoprotein in the pathogenic bloodstream stage. Also critical to virulence is the endo/lyosomal system, which is upregulated in bloodstream parasites for uptake and degradation of host serum proteins. N-glycosylation in trypanosomes is a uniquely stage-specific process. Insect stage parasites transfer only triantennary Man GlcNAc, while bloodstream parasites also transfer biantennary paucimannose Man GlcNAc to defined sites. Transfer of these classes 9 2 5 2 of N-glycans is mediated by two oligosaccharyltransferases with distinct preferences for glycan structure: STT3B is constitutively expressed and favors oligomannose, STT3A is bloodstream specific and favors paucimannose. Oligomannose glycan synthesis can be blocked by Man GlcNAc, but due to the lack of 5 2 Golgi manniosidase II cannot be further processed. However, paucimannose oligosaccharides can be trimmed and extended to simple asialobiantennary oligosaccharides, as well as hyper-processed poly-N-acetyllactosamine (pNAL) stuctures. pNAL is reactive with tomato lectin (TL), and is typically found on glycoproteins of the parasite lyso/endoosomal pathway, including the transmembrane glycoprotein p67. A bloodstream stage specific protein disulfide isomerase (TbPDI2) has also been characterized as a pNAL-containing lysosomal enzyme (JBC, 2005, 280:10410), despite having a proper C-terminal ER retention signal (JBC, 1996, 271:18378). We find that TbPDI2 is TL-reactive, but is an ER resident glycoprotein that does not contain pNAL. Using a combination of sequential precipitation with antibody (anti-p67 or anti-TbPDI2) and lectins (TL or ECL) we demonstrate that paucimannose containing glycopolypeptides are reactive with TL within minutes of biosynthesis, and well before transport to the Golgi, suggesting an unexpected interaction of TL with the limiting mannochitobiose core. Consistent with this interpretation, jackbean α-mannosidase treatment converts the oligomannose glycans of TbCathepsinL to TL-positive reactivity. Formal proof that TL reacts with paucimannose glycans will require abrogation of TbSTT3A expression. This work resolves the contradictory status of TbPDI2 and serves a cautionary note about the use of TL for determination of N-glycan structure.
The oocyst walls of Toxoplasma have an inner layer of β-1,3-glucan (like fungi) and an outer layer of acid-fast lipids (like mycobacteria).

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Toxoplasma gondii is a protozoan parasite that is infectious to humans and other animals with the cat being the primary host. The oocysts of Toxoplasma gondii are shed in cat feces and after ingestion can cause severe infections in fetuses and in patients with AIDS. While numerous proteins have been identified in oocyst walls, there is no model for its structure. Here, we show that the inner layer of the oocyst wall of Toxoplasma is a porous scaffold of fibrils of β-1,3-glucan, the sugar polymer present in fungal walls. While glucan synthase inhibitors (echinocandins) kill fungi, these drugs arrest the development of oocyst walls of Eimeria, a chicken parasite that closely models Toxoplasma oocysts. The outer layer of the oocyst wall of Toxoplasma gondii contains acid-fast lipids like those that cover the surface of mycobacteria. Organic solvents dissolve the outer layer of the oocyst wall and release triglycerides with polyhydroxy fatty acyl chain like those of the waxy cuticle of plants. The oocyst wall of Cryptosporidium parvum, a major cause of diarrhea and death in infants in the developing world, contains acid-fast lipids but does not contain glucan fibrils. Our characterization of β-1,3-glucan and acid-fast lipids in oocyst walls could represent a major breakthrough in the understanding of how these parasites cause disease.

Galectin-3 is a damage-associated molecular pattern molecule, which facilitates neutrophil recruitment as an innate immune response to a parasitic protozoa cutaneous infection

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Series of recent works suggest that galectin-3 could modulate immune responses as a lectin by recognizing glycans attached on the surface glycoproteins. Importantly, however, galectin-3, like other members of galectin family, is synthesized as a cytosolic protein and stored in the cytosol, segregated from its glycan ligands, which exist in the extracellular space. While it remains elusive how galectins are secreted actively by stromal cells, including leukocytes, some reports indicate that galectin-3 is passively released by damaged cells. Immediately after the infection, the innate immune system senses the invading microorganism and to estimate the virulence, which would be introduced to the host by the microbe (such as tissue or cell damage) in order to initiate host immune responses. Thus, it is possible that released galectins from damaged tissues could be one of such molecules, recognized by innate immune system. One of the innate responses is the rapid migration of neutrophils to the affected site. While neutrophils neutralize microorganisms, they can also cause tissue damage or render invasion pathways to pathogens. To investigate whether galectin-3 could act as an early immunomodulator in the initial stage of infection, we investigated the role of galectin-3 in neutrophil migration and the biological significance of the rapid migration of neutrophils in an experimental parasitic cutaneous infection with Leishmania major. When the substrain of L. major, LV39, was infected, lack of galectin-3 impaired neutrophil recruitment in the footpads and the draining lymph nodes one day following infection. Reduced number of recruited neutrophils correlated with local high parasite burdens. In contrast, neutrophil migration, induced by the other L. major substrain, Friedlin, was unaffected and the initial parasite burden remained similar in galectin-3 null mice as compared to wild type mice. Infection with L. major LV39 but not Friedlin induced higher levels of extracellular release of galectin-3. Further, galectin-3 alone was able to initiate neutrophil migration even though galectin-3 is not a chemoattractant for neutrophils. Thus, our data suggest that once extracellularly released, galectin-3 can act as a DAMP, damage-associated molecular pattern, to facilitate early neutrophil migration, which is beneficial in the initial control of the Leishmania infection.

Immune Regulation by Glycans

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The hygiene hypothesis states that the changes in microbial exposure associated with the increasingly sterile Western environment is the root cause for the dramatic increases in allergy and autoimmunity over the last several decades. Another factor that is believed to be important is the increased use of vaccines, which prevent infectious disease while eliminating the potentially beneficial immunologic education that comes from such exposures. Our research has focused on one potential player within this context, the commensal bacterium Bacteroides fragilis and its capsular polysaccharide PSA. This and other “glycoantigens” are gaining recognition as potent immunomodulatory factors that help to not only direct the maturation of the immune system, but also to limit pathologic inflammation associated with disease. Most recently, we have demonstrated that PSA stimulates a protective T cell-dependent response from the gut that can influence peripheral tissues, such as the lung and airway, and their responses to pro-inflammatory signals through a novel mechanism. For example, PSA elicits strong anti-inflammatory T cells that prevent the onset of experimental asthma. This presentation will highlight our published and unpublished data that collectively establishes commensal polysaccharide antigens as critical features underlying the hygiene hypothesis.
Chitin is an essential structural polysaccharide of fungal pathogens and parasites, but its role in human immune responses remains largely unknown. It is the second most abundant polysaccharide in nature after cellulose and its derivatives today are widely used for medical and industrial purposes. Small fungal chitin particles derived from Candida albicans led to selective secretion of the anti-inflammatory cytokine IL-10. We identified NOD2, TLR9, and the mannose receptor as essential fungal chitin-recognition receptors for this response. Chitin reduced inflammation in vivo and may contribute to the resolution of the immune response once the pathogen has been defeated. Fungal chitin also induced eosinophilia, underpinning its ability to induce asthma. Moreover, polymorphisms in the identified chitin receptors, NOD2 and TLR9, predispose individuals to inflammatory conditions such as Crohn’s disease. Chitin recognition is therefore critical for immune homeostasis and is likely to have a significant role in infectious and allergic disease.

Galectins constitute an evolutionarily conserved family of β-galactoside-binding proteins found in the cytosol, nucleus, and the extracellular space. They are classified into three major structural types: (i) proto-type galectins contain one carbohydrate recognition domain (CRD) and form homodimers; (ii) chimera-type galectins have a single CRD and may oligomerize forming trimers and pentamers; (iii) tandem-repeat-type galectins are comprised of two CRD joined by a linker peptide. All three major galectin types described in mammals, “proto”, “chimera”, and “tandem-repeat”, are present in zebrafish, and phylogenetic topologies confirm the expected clustering with their mammalian orthologues. Galectins bind endogenous (“self”) galactose-containing complex carbohydrate and mediate a variety of biological processes, such as early development, tumor evasion, chronic inflammatory diseases and autoimmunity. In recent years, accumulating evidence has revealed that galectins are emerging as pattern recognition receptors in innate immunity by interacting with “non-self” microbial surface glycans. In this study, we used the zebrafish (Danio rerio) and its pathogen, the infectious hematopoietic necrosis virus (IHNV), as a model to assess the potential role(s) of the host’s galectins in viral recognition and infectivity. Our preliminary results suggest that zebrafish galectins (Drgal-1, Drgal-3 and Drgal-9) interact directly with the IHNV glycosylated envelope in a carbohydrate dependent manner as well as with the endogenous galactose-containing glycans. Furthermore, galectins have bivalent or multipotent binding activities, which modulate the viral adhesion to the host by bridging the IHNV to the surface of the host. 

Human milk uniquely possesses a rich pool of free-reducing glycans whose functions and bioactivity are not well understood. To explore the functional glycobiology of human milk glycans (HMGs), we generated a HMG shotgun glycan microarray (SGM) from 10 milk donors. Using the HMG-SGM, we interrogated the HMG-SGM with lectins and antibodies, which provided significant clustering with their mammalian counterparts, forming the expected clustering with their mammalian glycans. In this study, we used the zebrafish (Danio rerio) and its pathogen, the infectious hematopoietic necrosis virus (IHNV), as a model to assess the potential role(s) of the host’s galectins in viral recognition and infectivity. Our preliminary results suggest that zebrafish galectins (Drgal-1, Drgal-3 and Drgal-9) interact directly with the IHNV glycosylated envelope in a carbohydrate dependent manner as well as with the endogenous galactose-containing glycans. Furthermore, galectins have bivalent or multipotent binding activities, which modulate the viral adhesion to the host by bridging the IHNV to the surface of the host.

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Roles of Heparan Sulfate in Normal and Cancer Stem Cells of the Intestine
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The epithelium of the intestine develops from stem cells residing in the crypt region. From these stem cells, enterocytes, mucus secreting cells, and endocrine cells are generated during the journey to the top of the villi. It has been shown that stem cells produce those non-stem cell populations while they maintain self-renewal capacity. To determine the roles of heparan sulfate in intestinal cell development, we utilized lgr5-driven inducible gene knockout, which was generated by Hans Clevers (Nature; 459: 262-265, 2009). After treatment on mouse with tamoxifen, heparan sulfate was significantly abrogated in particular in jejnum. As a result height of villus was shorter than EXT1wt/ wt, and this was associated with fewer enterocyte and endocrine cells. While MUC2 positive goblet cells were not apparently affected, the number of goblet cells increased. Surprisingly, ki-67 staining was diminished in crypt cells of EXT1 knockout, suggesting that intestinal stem cells require heparan sulfate in stem cells proliferation, and this leads to lower number of differentiated enterocytes and endocrine cells. In EXT1 knockout mouse, azoxymethane (AOM) and dextran sodium sulfate induced adenoma while adenocarcinomas were apparently produced in wild-type mouse counter parts. Moreover lgr5-driven heterozygous elimination of APC tumor suppressor gene resulted in the formation of adenocarcinoma cell. However formation of adenocarcinoma was abrogated when EXT1 gene was also inactivated. The results strongly suggest that cancer stem cells also require heparan sulfate to produce progenies. Further work is critical to determine the role of heparan sulfate in colon cancer formation, possibly by utilizing in vitro organoid culture system. The work is support by NCI grant, P01CA71932

Intramural hypoxia induces sialyl-Tn antigen expression and facilitates tumor metastasis
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Intramural angiogenesis sustains unlimited tumor proliferation by supplying nutrition and oxygen and by maintaining intratumoral microenvironments, though extensive tumor cell proliferation causes insufficiency of oxygen, so-called "hypoxia". Intratumoral hypoxia is a pathological condition highly associated with tumor metastasis and progression, although the detailed molecular mechanism has not been well elucidated. In vivo pulse-chase labeling of hypoxic region of tumor tissues revealed that intratumoral migrating cells against proximal blood vessels appeared in hypoxic area, which specifically expressed Sialyl-Tn (sTn) antigen. We identified that the sTn antigen was synthesized by ST6GalNAc-I glycosyltransferase, the expression of which was transactivated by hypoxia-inducible factor (HIF-1) under cellular hypoxic conditions. Inconsistent with these findings, human clinical lung carcinoma tissues exhibited substantial expression of sTn antigen in hypoxic area. We have recently revealed that sTn antigen stimulates TGF-β production in tumor-associated macrophages through Siglec-15 signaling. This initiates Epithelial-Mesenchymal Transition thought to enable the dissemination of tumor cells into the surrounding tissues. We, furthermore, found that production of sTn antigen significantly elevated autonomous cellular motility. This was coincident with sTn-glycosylation of collagen receptor, αβ1 integrins, and enhanced activation of focal adhesion kinase that consequently enhanced cellular collagen binding and facilitated collagen-mediated cellular invasion. Monitoring blood circulating tumor cells in tumor-transplanted mice revealed that sTn antigen expression facilitated tumor intravasation that was suppressed by genetic inactivation of ST6GalNAc-I, or α2 integrin or by cellular treatment with monoclonal antibodies against sTn antigen. These results indicate that intratumoral hypoxia-induced sTn antigen is a functional molecule, which modulates intratumoral immunological environments and facilitates intratumoral invasion and intravasation that consequently facilitates hematogenous tumor metastasis. Indeed, sTn antigen is a clinical tumor marker, the expression of which is well associated with tumor metastasis and poor prognosis. This study provides novel pathophysiological insights into the mechanism of the hypoxia-induced tumor metastasis and contributes to develop new anti-metastasis drugs by targeting tumor associated glycan functions.

Harnessing Novel Biomarkers of Human Embryonic Stem Cells for Cancer Diagnosis and Therapy
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Human embryonic stem cells (hESCs) are defined as a group of cells at the preimplantation stage of embryo, with the capacity for self-renewal and differentiation to generate different types of cells and tissues. Cancer stem/initiating cells (CSC) also possess the capability of stem cells to multiply and differentiate into their progenies, display resistance to chemotherapy and radiation therapy, and could be the root cause for relapse and metastasis of cancerous tumors. On the cell surface, more than 85% of proteins are glycosylated. Recent studies indicated that glycosphingolipids (GSLs) are ubiquitous components of cell membranes and, similar to surface glycoproteins, can act as mediators of cell adhesion and signal transduction. Therefore, a systematic survey of expression profiles of GSLs and
glycoproteins in hESCs and various differentiated derivatives was carried out. Based on MALDI-MS and MS/MS analyses, we have found a number of unique expressions of GSLs in the undifferentiated hESCs and induced pluripotent stem (iPS) cells, and also a close association of the GSL expressions in hESCs and iPS cells with differentiation. On the other hand, Globo H, a known biomarker for cancers, was highly expressed uniquely in undifferentiated hESCs and iPS cells. TJs and other ESC signatures unique for hESCs and iPS cells will perhaps be the targets of therapy for cancer. In addition, we had also employed glycoproteomics and glycan analysis to analyze the expression of sialylated N-glycoproteins for hESCs. We have identified seven newly found surface N-linked sialoglycoproteins that are expressed abundantly in hESCs prior to differentiation and their expression levels are many folds higher in breast cancer CSC as compared to non-CSC. For example, silencing of ESC02 leads to decrease in cell proliferation of hESCs and mammary sphere formation of breast cancer, leading to cell arrest. In addition, loss-of-function of ESC02 results in developmental skewing toward endoderm/mesoderm differentiation in vitro and in vivo. These findings warrant the development of ESC02 as target for new therapy. Furthermore, ESC02 sheds to the plasma, resulting in higher level of auto Abs detected in patients with breast cancer. The area under receiver operating characteristic curve (ROC) for these auto Abs in patients was 0.86, indicating excellent discrimination for cancer diagnosis. Therefore, these newly found GSLs and glycoproteins present in hESCs and iPS cells could be candidates for cancer diagnosis and glycan-targeted therapy of tumors.

(46) Heparan Sulfate Is Required For Prostate Cancer Initiation And Progression
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Prostate cancer (PCA) is one of the most prevalent forms of malignancy and the second most common cause of cancer-related death in men. The failure in treatment of this disease is our inability to prevent and control PCA growth and metastasis. A better understanding of the mechanisms underlying PCA pathogenesis will greatly enhance our effort to cure this life-threatening disease. Heparan sulfate (HS) is a linear, sulfated polysaccharide, and expresses abundantly in prostate and PCA tissues. Intriguingly, the HS content and sulfation modifications appear to increase when the prostate becomes malignance, suggesting that HS may critically modulate PCA pathogenesis. In current study, we specifically ablated Ext1, the enzyme that initiates HS biosynthesis, in mouse prostate at late development stage. The Ext1 ablation does not affect prostate development and function, instead, it protects the mice from tumorigenesis and invasion in a spontaneous PCA mouse model. Tissue staining showed that the Ext1 deficiency attenuated PCA cell proliferation, increased apoptosis, and blocked PCA stem/progenitor cell differentiation and epithelial-mesenchymal transition. The Ext1 deficiency PCA tissues also showed significant attenuation of fibrinosis, MMP-9 expression and hypoxia. In summary, our studies demonstrate that HS functions via multiple mechanisms to promote PCA tumorigenesis and invasion, and also reveal that targeting HS may represent a novel and effective approach to cure PCA.

(47) O-GlcNAc modification of ribosomal RACK1 enhances hypoxia-induced epithelial–mesenchymal transition in hepatocellular carcinoma
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Hepatocellular carcinoma (HCC) is among the most common and aggressive cancers worldwide, and hypoxia plays a critical role in proliferation, angiogenesis, metastasis and survival of HCC. Our previous research has indicated that ribosomal RACK1 promoted the chemoresistance and growth in HCC. In this study, we found that ribosomal RACK1 enhanced hypoxia-induced epithelial–mesenchymal transition in HCC, and this effect was dependent on the O-GlcNAc modification of RACK1 at the site of Serine 122. Hypoxia promoted S122 O-GlcNAcylation of ribosomal RACK1, leading to its enhanced stabilization and ribosome association. Ribosomal RACK1 stimulated the translation of Snail1 and Twist as well as epithelial–mesenchymal transition under hypoxia in S122 O-GlcNAcylation-dependent manner. Our results indicate that O-GlcNAcylation of ribosomal RACK1 enhances hypoxia-induced epithelial–mesenchymal transition in HCC, and provide evidence to link O-GlcNAc modification to translational regulation and HCC metastasis.

(48) Syndecan-coupled receptor kinase signaling in Angiogenesis
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The syndecans are matrix receptors with roles in cell adhesion and growth factor signaling. Their heparan sulfate chains recognize “heparin-binding” motifs ubiquitously present in the ECM, providing the means for syndecans to constitutively bind and cluster to sites of cell-matrix adhesion. Work in our laboratory suggests that specialized docking sites in the syndecan extracellular domains serve to localize other receptors to these sites as well, including integrins and growth factor receptor tyrosine kinases. The prototype of this mechanism is the capture of the αvβ3 integrin and insulin-like growth factor-1 receptor (IGF1R) by syndecan-1 (Sdc1) that form a ternary receptor complex in which signaling downstream of
the IGF1R generate the inside-out signal leading to activation of the integrin, and is also critical for cell survival. VEGF-stimulated angiogenesis depends on a poorly understood cross-talk mechanism involving VEGF receptor 2 (VEGFR2), vascular endothelial (VE)-cadherin and the αVβ3 integrin. We find that the Sdc1-coupled ternary receptor complex is required for VEGF signaling and for stimulation of vascular endothelial cell migration by VE-cadherin engagement. Plating endothelial cells on Fn/VE-cadherin chimera activates Sdc1-coupled IGF1R and αVβ3 integrin; this depends on VEGFR2 and c-Src activated by cadherin engagement. Blocking homotypic VE-cadherin engagement disrupts VEGF-stimulated cell migration, which is restored by clustering the cadherin in the absence of cell-cell adhesion. This cadherin-dependent stimulation requires VEGFR2 and IGF1R and is blocked by synstatin (SSTN 92-119), a peptide that competitively disrupts αVβ3 integrin; this depends on VEGFR2 and c-Src activated by cadherin engagement. Blocking homotypic VE-cadherin engagement disrupts VEGF-stimulated cell migration, which is restored by clustering the cadherin in the absence of cell-cell adhesion. This cadherin-dependent stimulation requires VEGFR2 and IGF1R and is blocked by synstatin (SSTN 92-119), a peptide that competitively disrupts αVβ3 integrin activation that is required for VEGFR2 activation. VEGFR2-stimulated angiogenesis in the mouse aortic ring explant assay is disrupted by SSTN, but only early in the process, suggesting that IGF1R coupling to Sdc1 and αVβ3 integrin comprises a core activation mechanism activated by VE-cadherin that is necessary for VEGFR2 and integrin activation during the initial stages of endothelial cell dissemination during angiogenesis.

(49) Glycosylation in the Metabolic Origins of Common Griefous Disease
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Center for Nanomedicine, SBMRI and UCSB

Glycosyltransferases and glycosidases represent much of the enzymatic machinery in cells that compose the structural repertoire of glycans, often termed the glycome. Changes in the glycome can be observed in both genetic and acquired disease states. While some genetic diseases have been linked to DNA sequence variation, multiple common diseases and syndromes appear to originate from metabolic changes in the absence of detectable genomic variation. From studies over the past two decades, this laboratory has identified environmental triggers that alter mammalian and human glycosylation and which include metabolic origins of common grievous diseases. These metabolic triggers include dietary stress and pathogen-host interactions. The disease pathways induced by such metabolic triggers include, for example, the pathogenesis of obesity-associated diabetes. In diabetes, an acquired change in N-glycosylation is responsible for disabling the first step of glycolysis among pancreatic beta cells, which then causes beta cell dysfunction that is linked to disease onset. In another example, the altered metabolism of glycans in the blood has been found to determine the outcomes of host infection by pathogens, in the absence of genomic variation among pathogen and host. Examples of such microbial interactions have been identified in our research that includes host responses to infection by Streptococcus pneumoniae and Salmonella Typhimurium. Infection by these microbes causes metabolic changes in host glycans that are sensed by host receptor systems that reside in various tissues. Host receptor responses then determine the outcome of disease, including the severity of host tissue damage and the frequencies of host survival. We are therefore developing high-throughput detection approaches to simultaneously interrogate the four major types of cell components, namely nucleic acids, proteins, lipids, and glycans. By interrogating both inherited and metabolic factors, research can more completely reveal the origins and pathways of common diseases that arise from metabolic triggers in the context of a normal or susceptible genomic background.

(50) Galectin-3 disruption impaired melanoma associated angiogenesis and reduced VEGF secretion from macrophages stimulated by TGFβ1
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Galectin-3 expression has been involved in a variety of tumor-related processes, including angiogenesis. Within tumors, parenchymal (tumor) cells express varying levels of galectin-3. On the other hand, tumor associated macrophages express high amounts of galectin-3. In order to study the role of galectin-3 in tumor angiogenesis associated with tumor associated macrophages (TAM) and tumor parenchyma, the galectin-3 expression was reconstituted in Tm1 melanoma cell line that lacks this protein. Galectin-3 expressing cells (Tm1G3) and mock-vector transfected cells (Tm1N3) were injected into wild type (WT) and galectin-3 knockout (KO) C57Bl/6 mice. Tumors originated from Tm1G3 were larger in tumor parenchyma, the galectin-3 expression was reconstituted in Tm1 melanoma cell line that lacks this protein. Galectin-3 expressing cells (Tm1G3) and mock-vector transfected cells (Tm1N3) were injected into wild type (WT) and galectin-3 knockout (KO) C57Bl/6 mice. Tumors originated from Tm1G3 were larger in tumor parenchyma, the galectin-3 expression was reconstituted in Tm1 melanoma cell line that lacks this protein. Galectin-3 expressing cells (Tm1G3) and mock-vector transfected cells (Tm1N3) were injected into wild type (WT) and galectin-3 knockout (KO) C57Bl/6 mice. Tumors originated from Tm1G3 were larger in tumor parenchyma, the galectin-3 expression was reconstituted in Tm1 melanoma cell line that lacks this protein.
Arginase-I protein levels and galectin-3 expression in WT-BMDC, but not in cells from KO mice. Hence, we report that galectin-3 disruption in tumor stroma and parenchyma decreases angiogenesis through interfering with the responses of macrophages to the interdependent VEGF and TGFβ1 signaling pathways. Supported by FAPESP (1998/14247-6)

(51) The role of glycansynthesis and degradation in platelet birth and death
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The role of glycansynthesis and degradation in platelet birth and death. Karin M. Hoffmeister Delayed platelet recovery and bleeding complications are major determinants of morbidity and mortality following cancer treatment. Major therapies to restore platelet counts and prevent bleeding complications are platelet transfusions and bone marrow (BM) stem cell transplants. Platelets are produced by megakaryocytes (MKs), which differentiate and mature from BM hematopoietic stem cells. Mature MKs are residents of the vascular BM niche and interact with sinusoidal BM endothelial cells to form transendothelial pseudopods, called proplatelets, which release platelets into the bloodstream. Following release, platelets circulate for 5 days in mice and 10 days in humans. The regulatory mechanisms of platelet birth and death remain elusive. In recent years it has become increasingly apparent that platelet life-span is regulated by glycan-lectin clearance mechanisms. Platelet surface glycans become degraded during in vitro storage/aging, i.e. platelet glycans undergo a significant decrease in sialylation and galactosylation and become cleared by hepatic Ashwell Morell receptors and αMβ2 lectin domains upon transfusion, severely diminishing post transfusion platelet survival and function. Now we have obtained direct evidence that lactosaminyl glycans (LacNAc) synthesized by the β4galatosyltransferase 1 (β4GalT1) not only regulate platelet life span, but are key regulators of thrombopoiesis. β4GalT1−/− mice exhibit severe macro-thrombocytopenia, and megakaryocytes lacking functional β4GalT1 fail to produce circulating platelets, although they mature and differentiate normally. Hematopoiesis depends on the interplay of temporally expressed adhesion molecules that regulate the vascular and osteoblastic niches in the BM. Expression of lactosaminyl glycans within hematopoietic marrow cells occurs in a stage-specific and lineage-specific fashion. Our data show that LacNAc synthesis mediated by β4GalT1 is crucial for platelet genesis, unrevealing a novel role for post-translational glycan modification in platelet birth.

(52) Heparosan polysaccharide for drug delivery; a biosuperior alternative to PEGylation
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Many drugs have delivery problems in the body. Currently, PEGylation, a FDA-approved process of adding poly[ethylene glycol] ‘vehicle’ to therapeutic ‘cargo’ including small molecules, polypeptides, and liposomes, is used to enhance drugs by protecting the cargo when in the body and prolonging therapeutic action. PEG, however, has some liabilities due to its artificial nature including potentially toxic degradation products, accumulation in the body, and its recently recognized immunogenicity. Heparosan is a linear polysaccharide that is a member of the glycosaminoglycan [GAG] family that includes hyaluronic acid [HA], heparin and chondroitin. Heparosan should be biocompatible because it is the natural unmodified precursor in the heparin biosynthetic pathway, and certain pathogenic bacteria use a heparosan coating as a molecular camouflage. We have harnessed a bacterial heparosan polymerizing enzyme called heparosan synthase, PmHS1, from Pasteurella multocida. This catalyst is useful for the chemoenzymatic synthesis of sugar polymers with a very narrow size distribution with defined chemical activation to facilitate monovalent, selective coupling to cargo. The heparosan chain enhances the pharmacokinetics of therapeutics by increasing their hydrodynamic radius and defending against proteolytic degradation. In addition, antibodies are blocked from binding to the cargo surface. A key advantage with heparosan when compared to other GAGs is that it has increased biostability in the extracellular space; depending on its size we have observed 16-192 hour half-life in the bloodstream of rodents and primates. However, if heparosan or any of its fragments is internalized by the cell and is transported to the lysosome, then these chains are degraded by resident glucuronidase and hexosaminidase enzymes just as for heparin or HA. We did not observe unwanted accumulation in any organ. The normal roles of heparin/ heparan sulfate in vertebrates include binding to coagulation factors, but heparosan, which lacks sulfate groups, does not affect clotting of human plasma even when used at much higher doses than heparin. We have also verified that the heparosan molecule is non-toxic in rats even at 100 mg/kg intravenous dosing. Overall, heparosan is a stealthy biocompatible polymer in mammals that has now been employed successfully to enhance several protein-based drugs.

(53) Ovarian tumor cell survival mechanisms regulated by ST6Gal-I sialyltransferase
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Survival rates for ovarian cancer (OC) have improved only incrementally despite decades of research, underscoring the need to identify novel disease-related molecular pathways. Our group has discovered that the addition of a2-6-linked sialic acids to selected receptors by the ST6Gal-I sialyltransferase endows OC cells with the capacity to avoid cell death induced by chemotherapy drugs and death receptor activators. In the first immunohistochemical (IHC) analyses of ST6Gal-I in OC, ST6Gal-I expression was found in the major OC subtypes, whereas ST6Gal-I was undetectable in uninvolved ovarian epithelium. In papillary serous adenocarcinoma, the most prevalent and lethal OC subtype, 32/33 tumors exhibited ST6Gal-I upregulation. Mechanistic studies suggest ST6Gal-I...
upregulation is a driving factor in OC resistance to platinum-based chemotherapeutics. Forced ST6Gal-I overexpression inhibits, while knockdown enhances, cisplatin-induced OC cell death, and OC cells selected for acquired stable cisplatin resistance have increased endogenous ST6Gal-I. These results implicate ST6Gal-I as a promising therapeutic targeting for restoring clinical response to platinum drugs. In other studies we determined that ST6Gal-I upregulation protects OC cells against apoptosis induced by the cytokine, TNFα, implicating a novel function for ST6Gal-I in escape from immune surveillance. ST6Gal-I sialylation of TNFR1, (receptor for TNFα), prevents TNFα-induced TNFR1 internalization, an event necessary for apoptosis. However, α2-6 sialylation does not simply shut off TNFR1, but instead diverts signaling toward pro-survival pathways directed by NFκB and MAPKs. This switch is accomplished by sialylation-dependent retention of TNFR1 at the cell surface. We posit that altered signaling by ST6Gal-I implicates a novel function for ST6Gal-I in escape from immune surveillance.

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Podoplanin (PDPN) is a mucin-type O-glycoprotein. We previously reported that core 1 O-glycosylation is critical for the function PDPN during the development of lymphatic vessels. Here, we report a role for PDPN in maintaining the barrier function of high endothelial venules (HEVs), which are specialized blood vessels essential for the transmigration of lymphocytes into lymph nodes. Circulating lymphocytes continuously enter lymph nodes for immune surveillance through HEVs, a process that increases dramatically during immune responses. How HEVs permit lymphocyte transmigration while maintaining vascular integrity is unknown. We found that mice with postnatal deletion of PDPN lost HEV integrity and exhibited spontaneous bleeding in mucosal LN, and bleeding in the draining peripheral LN after immunisation. Blocking lymphocyte homing rescued bleeding, indicating that PDPN is required to protect the barrier function of HEVs during lymphocyte trafficking. Further analyses demonstrated that PDPN expressed on fibroblastic reticular cells (FRCs), which surround HEVs, functions as an activating ligand for platelet C-type lectin-like receptor 2 (CLEC-2). Mice lacking FRC PDPN or platelet CLEC-2 exhibited significantly reduced levels of VE-cadherin (VE-cad), which is essential for overall vascular integrity, on HEVs. Infusion of wild-type (WT) platelets restored HEV integrity in CLEC-2-deficient mice. Activation
of CLEC-2 induced release of sphingosine-1-phosphate (S1P) from platelets, which promoted expression of VE-cad on HEVs ex vivo. Furthermore, draining peripheral LNs of immunised mice lacking S1P had impaired HEV integrity similar to PDLPN-and CLEC-2-deficient mice. These data demonstrate that local S1P release after PDLPN-CLEC-2-mediated platelet activation is critical for HEV integrity during immune responses.

(56) Insight into UDP-GlcNAc : lysosomal enzyme
N-acetylglycosamine-1-phosphotransferase function/ localization via analysis of mutations in Mucolipidosis II/ III
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UDP-GlcNAc : lysosomal enzyme N-acetylglycosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase) is an α/β heterohexamer that 2 2 mediates the initial step in the formation of the mannose 6-phosphate recognition signal on lysosomal acid hydrolases. Mutations in the GNPTAB gene that encodes the α/β subunit precursor give rise to the lysosomal storage disorders Mucolipidosis II and III α/β that are characterized by hypersecretion of newly synthesized acid hydrolases. We are analyzing the consequence of missense mutations in this gene with the goal of gaining insights into the function of the various domains of GlcNAc-1-phosphotransferase. 5In an initial screen, HEK293 cells transfected with the various mutant cDNAs were analyzed for proteolytic processing of the α/β precursor to the α and β subunits, a step that occurs in the Golgi. Mutations that impaired folding in the endoplasmic reticulum were excluded (9/27). Studies of the effects of the remaining mutations on Golgi localization, catalytic function, recognition of lysosomal acid hydrolases as specific substrates and turnover were then initiated. To date, we have documented that: (1) a mutation in the DMAP interaction domain of the α subunit (K732N) impairs phosphorylation of acid hydrolases without decreasing catalytic activity toward the simple sugar α-methylmannoside, implicating the DMAP domain in the selective recognition of acid hydrolase substrates. (2) Two missense mutations in the N-terminal cytoplasmic tail of the α subunit (K4Q and S15Y) cause the loss of localization in the Golgi and rapid degradation in lysosomes indicating a critical role for the cytoplasmic tail in Golgi retention/ recycling. We conclude that this is a valuable approach for elucidating the function of this critical enzyme.

(57) The Big Bang: An Expanding Universe of Human Glycosylation Disorders
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In 2013, a new human glycosylation disorder was reported on average every 11.7 days. Additional genes affecting glycosylation pathways or alter glycan structure will certainly be identified. The clinical phenotype of patients with function-compromising mutations in these genes demonstrates their importance, health relevance, and it creates novel research opportunities. This discovery bonanza occurred from a coalescence of genomic, biochemical and social media technologies for all stakeholders: scientists, physicians, patients themselves, and their families. Efficient exome/genome analysis will soon become a clinical diagnostic test to identify a few candidate genes, but they require functional confirmation. Sophisticated informatics can dismiss critical candidates. For example, we found spontaneous mutations the UDP-Gal transporter, SLC35A2, in 6 patients. Standard exome analysis rejected the candidate, but routine analysis of a glycan-based biomarker, transferrin, over ruled gene analysis. Biochemical analysis and exome/ genome sequencing enabled us to identify 8 new glycosylation disorders from 50 patients who showed abnormal glycosylation. Also, exome sequencing of patients without prior glycosylation marker testing revealed new glyco-candidates that were validated by analysis of serum, fibroblasts or lymphoblasts. Families dealing with undiagnosed disorders scour the web and social networks daily. Our laboratories are a click away from their questions and their cooperation. Among the 3500 unsolved genetic disorders, some will alter glycosylation, and the basic scientist can lead the assault on functional analysis of these genes with support from an increasingly savvy group of web-wise families. This creates an opportunity to educate and strengthen the vital and expanding links between basic scientists, physicians and the voter/ taxpayer families. Solving glycosylation disorders provides a model of how to integrate and advance both medical and fundamental science to benefit affected families. Supported by The Rocket Fund.

(58) The colon mucus system and the interaction of the MUC2 mucin O-glycans with the microbiota in relation to evolution and colitis
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MUC2 is the major gel-forming mucin of the colon forming a protective gel barrier organized into an inner stratified and an outer loose layer. The MUC2 N-terminus (D1-D2-D3 domains) has a dual function in packing the MUC2 polymer in the goblet cells into an N-terminal concatenated polygonal platform with the C-terminals extending perpendicularly by pH-and calcium-dependent interactions and by building a net-like structure by disulfide-bonded trimerization after secretion (Ambort et al. 2012). The mucin domains of MUC2 are densely decorated with O-glycans. These glycans are important for generating unfolding forces for the correct expansion of the MUC2 mucin and to inhibit degradation of the mucin polymer. The MUC2 O-glycosylation varies along the GI-tract, and the specific structures on the mucin domains in the MUC2 of colon are for mucin unusually uniform between different human individuals. In addition to the mucin domains, additional sites are glycosylated on the MUC2 mucin by both N-and O-glycans. Of special importance is O-glycosylation at a specific site susceptible to proteolysis within the MUC2 mucin. Both the parasite Entamoeba histolytica and the bacterium Porphyromonas gingivalis has developed specific proteases that are able to cleave at this site when a specific Thr is not glycosylated (van der Post et al. 2013). Studies have shown that only the
GalNAcT3 is able to add a GalNAc at this site. Cleavage at this site dissolves the protective inner colon mucus layer. Loss or defects of the inner mucus layer allows bacteria to reach the colon epithelium, something that triggers inflammation. The glycosylation of MUC2 is thus instrumental for protecting the colon from colitis (Johansson et al. 2013).

References


(59) Immune inhibitory and stimulatory glycans controlling tumor immunity
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Dendritic cells (DC) are specialized in the recognition of antigen and play a pivotal role in the control of immunity and tolerance. For antigen recognition DC express several C-type lectins, that function as innate receptors that facilitate antigen uptake and presentation. Many of these receptors also modify responses through signalling interference with TLR. We and others have shown that the C-type lectins DC-SIGN, MGL and Siglecs recognize specific glycan structures on pathogen related antigens but also on tumors. We have identified glycan structures that can either contribute to the tumor suppressive programming of the immune system, or oppositely activate the immune system. By modifying antigens with specific glycans we can favour directional targeting of tumor antigens to human DC and Langerhans Cells (LC) in situ, in the skin, facilitating the induction of tumor specific CD4 and CD8 T cell responses and Th1 differentiation. Therapeutic vaccination of mice with glycan modified antigen show long term anti-tumor immunity when tumor induced T regulatory cells are temporarily reduced. Moreover, we have identified key glycans expressed on tumor cells that contribute to the induction of immune regulatory processes. All together our work sheds new light on the contribution of glycans to control tumor immunity by either contributing to the induction of anti-cancer immunity or tumor induced immune suppression.

(60) Analytical Services and Trainings at the Complex Carbohydrates Research Center
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The CCRC Analytical Services Laboratory at The University of Georgia is a non-profit entity that offers services for structural characterization of glycoconjugates derived from animal, plant or microbial origin. Our scientists have many years of experience with challenging projects in the area of Glycobiology. The service laboratory is complimented with state-of-the-art instruments such as LTQ-Orbitrap-MS, AB 5800 MALDI-TOF/TOF-MS, high field NMR, HPLC, HPAEC, and GC-MS. We present examples of glycoconjugate analyses that apply a combination of techniques. Briefly described below are some analyses for each class of glycoconjugate. Glycoproteins: release of N- and O-linked glycans from purified, cells or gel-prepared glycoproteins, mapping N- and O-linked glycosylation sites on peptides, identification of type of N-linked glycans, i.e. biantennary, triantennary, tetraantennary, high mannoside, hybrid or complex, determination of residue, linkage composition and their sequence in oligosaccharides, ring size, anomic configuration, and determination of points of attachment, composition, and linkage of non-carbohydrate constituents such as phosphate and sulfate. Glycosaminoglycans: isolation from cells and tissues, disaccharide analysis, quantitation of HS/CS/HA, CTA-SAX, molecular weight determination, degree of sulfation, isolation and sequencing of individual oligosaccharides by MS and NMR. Polysaccharides: purification to homogeneity by SEC and/or ion exchange chromatography, and determination of glycosyl composition, linkage, ring size, and anomic configuration. Lipopolysaccharides (LPS): isolation of LPS from bacteria cells, release of Lipid A from O-antigen, and characterization of Lipid A by MS and O-antigen by techniques used for polysaccharides. Lipids and glycolipids: determination of fatty acids composition of cerebrosides, diacylglycerol or triacylglycerol, and phospholipids. We also offer annual hands-on training courses for structural characterization of both glycoproteins and polysaccharides.

(61) Strategies for Glycomics and Glycosaminoglycan Analysis as part of Analytical Services at the Complex Carbohydrate Research Center
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The Analytical Services Laboratory of the Complex Carbohydrates Research Center (CCRC) at The University of Georgia is a non-profit entity that offers services for structural characterization of glycoconjugates derived from animal, plant, or microbial origin. This poster highlights some of the diverse collaborative projects embarked over the past year. Glycan analysis of HEK-B4T Cells: Gal-free knockout (GTKO) porcine organs eliminated the dominant xenogeneic antigen galactose alpha 1,3, galactose (Gal) but they have not eliminated antibody mediated rejection. Antibody which
binds to GTKO endothelial cells (non-gal antibody) and vascular anti-body deposition is commonly reported at rejection of GTKO hearts. To further identify porcine antigens which induce non-Gal anti-body responses, Byrne et al. (1) identified the porcine β1,4 N-acetylgalactosaminyltransferase (B4GALNT2) gene by using cardiac xenotransplant sensitized primate serum to screen a porcine endothelial cell expression library. Porcine B4GALNT2 gene-transfected human HEK cells (HEK-B4T) show increased surface binding of anti-Sd antibody, and lectin Dolichos bilius agglutinin indicating increased terminal N-acetylgalactosamine expression. CCRC collaboration found glycosylation of HEP and HEK-B4T cells with NSI-FTMS analysis and MS/MS found both HexNAc enrichment N-glycans and O-linked Sd structures in HEK-B4T cells. Glycosylation of Plant-Produced Asialoerythropoietin: Although the desialylated form of human erythropoietin (EPO) shows great therapeu-tic potential in a number of applications, its use is currently hindered by the high costs of production through enzymatic desialylation of recombinant human EPO. Work by Xie et al. utilized a plant-based expression system to product soluble, fully functional EPO in high levels in transgenic tobacco plants. Detailed N-glycan analysis of tobacco produced asialo-rhEPO was conducted at the CCRC using NSI-FTMS and MS/MS studies revealed 13 different N-glycan chains. Chondroitin/ Dermanatan Sulfate Analysis of WHOI/ WHOIV Brain Carcinoma: Glycosaminoglycans isolated from WHO-I and WHO-I brain tumor tissue samples were subjected to enzymatic digestions with Chondroitinase ABC and AC to determine amounts of component disaccharides making up Chondroitin and Dermatan sulfates. Disaccharides were separated using SAX-HPLC coupled with post-column fluorescence labeling, for identification as well as quantification. A noticeably higher amount of sulfated disaccharides were found in the WHO-IV grade tumor in comparison to all six of the WHO-I tumors.

(62) Repository of recombinant expression constructs for mammalian glycosylation enzymes: production of glycosyltransferases and glycoside hydrolases in mammalian cells

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Glycan biosynthetic and catabolic enzymes play critical roles in encoding the diverse collection of carbohydrate structures involved in biological recognition events in animal systems. Mammalian glycosyltransferases (GTs) (~204 members) and glycoside hydrolases (GHs) (~75 members) are responsible for the assembly and degradation of glycan structures attached to intracellular and secreted proteins and lipids, yet the enzymatic, biochemical, and structural characteristics of these enzymes are generally not well understood and most are not available in sufficient quantities for biochemical studies or enzymatic synthesis of glycan structures. Challenges for recombinant production of the glycosylation enzymes arise from general requirements for eukaryotic expression to provide chaperone systems and post-translational modifications necessary for effective folding and function. To address the need for these recombinant products, we established a repository of expression constructs encoding the catalytic domains of all human GTs and GHs for production in HEK293 cells, baculovirus/insect cells, or in E. coli hosts. In mammalian cells, we developed an efficient transient transfection strategy for serum-free HEK293 suspension cultures providing secretion of the recombinant products into the conditioned media as affinity tagged forms. Alternative vector platforms with either short epitope and affinity tags or larger GFP or GFP-Fc fusions provide strategies for protein production, affinity purification, and protein detection and quantitation. Strategies for tag removal and glycan cleavage via combined protease and endoglycosidase digestion have also been developed to produce enzymes compatible with biochemical and structural studies. In addition, strategies for Se-methionine incorporation have been developed for SAD phasing of diffraction data from crystals of the purified proteins. The poster will summarize the status of construct generation and protein expression in mammalian cells as well as a comparison of expression data using the various fusion protein strategies. Scalability of the expression platform will also be demonstrated for the production of several GTs for structural studies. The availability of the constructs from the repository will also be summarized. The ultimate goals of the repository are to provide recombinant platforms for production of active glycosylation enzymes for use in biochemical, chemo-enzymatic, and structural studies on these critical human proteins. Supported by NIH Grants RR-005351 and GM103390.

(63) Repository of Recombinant Expression Constructs for Mammalian Glycosylation Enzymes: Baculovirus Vectors for Glycosyltransferase and Glycoside Hydrolase Production in Insect Cells

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Mammals encode ~204 glycosyltransferase (GT) and ~75 glyco-side hydrolase (GH) family members that assemble and degrade the diverse array of glycans attached to intracellular and secreted proteins and lipids, which have critical roles in biological recognition. To enable structural and functional studies on these enzymes, we have assembled a repository of recombinant plasmids and baculovirus expression vectors encoding most known human GTs and GHs for production in HEK293 or insect cells, respectively. These eukaryotic hosts have the chaperones and post-translational processing machinery needed for proper folding and enzymatic activity. Our original baculovirus vector design for all GT’s with N-terminal transmembrane domains used the polyhedrin promoter to express affinity tagged, secreted GT ectodomains, consisting of the stem and catalytic domains, and replaced the cytoplasmic and TM domains.
with an insect-specific signal peptide, 8X HIS and Strep-tag II affinity tags, and the Tobacco Etch Virus (TEV) protease cleavage site. Our original baculovirus vector design for all GH’s used the polyhedrin promoter to express each full-length protein with a C-terminal TEV cleavage site followed by the 8X HIS and Strep-tag II tags. During the course of the project, we modified this plan to include expression of both N- or C-terminally tagged versions of some GT’s, expression of some N-terminally tagged "glycan-related" enzymes, and constructed four additional baculovirus vectors designed to express GTs with different transcriptional promoters and/or purification tags for comparative purposes. This poster highlights some results obtained to date. In summary, we have expressed a total of 292 human glycoenzymes, including 152 different GT’s (20 as both N- and C-terminal constructs), 56 GH’s (30 as both N- and C-terminal constructs), and 34 glycan-related enzymes (all N-terminal constructs) in the baculovirus/insect cell system. Our analysis of the impact of using different promoters and purification tags on GT expression and secretion revealed no clear trend. The poster will include detailed maps of our expression constructs, production and secretion results obtained with a test set of sialyltransferases, and a comprehensive Table documenting the expression and secretion results obtained for all glycoenzymes expressed to date. Supported by NIH P41GM103390 and P41RR005351.

(64) Analyzing the modification of the Shewanella oneidensis MR-1 flagellar filament
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Shewanella oneidensis is a bacterium that is sometimes referred to as Shewanella oneidensis “MR-1”, indicating the “manganese reducing” capability of this organism. Species of the genus Shewanella belong to the group of facultatively anaerobic gammaproteobacteria, which exhibit a pronounced respiratory flexibility that allows them to use a wide range of alternative electron acceptors when growing under anaerobic conditions, including numerous metal ions, radio-nuclides and halogenated compounds. Thus, Shewanella species are thought to be suitable organisms for applications in bioremediation of contaminated soils or production of microbial fuel cells. The unsheathed flagellar filament of Shewanella oneidensis MR-1 is composed of two highly homologous flagellins, FlaA, and the major structural subunit, FlaB. We identified a gene cluster, SO_3261-SO_3265 (now smfABCDE), that is required for the formation of a fully functional filament and for motility. Intriguingly, loss of smfABCDE results in a significant mass shift of both FlaA and FlaB. Various mass-spectroscopy analyses were used to determine the site and the chemical nature of the putative post-translational modifications on FlaA and FlaB. The mass spectroscopic analyses and genetic analyses have identified at least four, more likely five, serine residues that are modified by O-linked glycosylation. The Q-TOF, NSI-CID and HCD -MS/ MS and NMR results indicated that the unknown modifications consist of a 274-Da moiety containing a nine-carbon keto-acid sugar with two nitrogens and one N-acetyl group. This residue is located at the reducing end of the glycan and is attached to serine. By considering the previous findings from other groups regarding flagellar modification, it is most likely a member of the pseudaminic-acid family. The 274-Da modification is further substituted with a 236-Da moiety, which can carry additional methyl groups (250 Da, 264 Da). In addition, our ETD-MS/ MS data indicated that at least 5 lysine residues in FlaB and one in FlaA are methylated. Based on homology comparisons we suggest that smfABCDE is required for species-specific flagellin modification in S. oneidensis MR-1.

(65) The N-linked Octasaccharide of M. mazei Does Not Resemble the Eukaryotic or Bacterial N-linked Counterparts
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Methanosarcina mazei, a major methane producer and strict anaerobic methanogen, belongs to the Archaea Methanosarcina family. Depending on the cell culture conditions, this branch of Archaea is well known for its unique diversity of forms: as single cells, bundles or lamina. In the absence of cell walls, its cells are blanketed by an outer glycoprotein envelope termed the S-layer (Surface-layer). Our prior Concanavalin A (ConA) labeled Epi-fluorescence microscopy studies showed only the aggregating cells interact with the lectin, suggesting that surface glycosylation plays a role in cell morphology. Glycoprotein and glycopeptide enrichment strategies combined with HCD MS/MS experiments and de novo sequencing allowed the identification of 38 unique glycopeptides from 23 different glycoproteins. All the glycopeptides contain a 1305-Da residual glycan with composition Hex dHex HexNAc. 5 2 After failed attempts to map the glycosylation site(s) by techniques widely used to determine the site of labile posttranslational modifications, e.g., ETD, ECD, and aiECD MS/ MS experiments, we employed hotECD MS/MS experiments. In seven unique glycopeptides, the hotECD MS/MS experiments unambiguously pinpointed the attachment site at a single Asn residue embedded within the classic N-linked consensus motif, NXT/S (X ≠ P). We have observed glycopeptides containing up to five unique glycosylation sites carrying a net glycan mass of 6597 Da. We also discovered glycan modifications such as acetylation and formylation on Hex residues. It is noteworthy that all the glycopeptides elucidated in this study contain an N-linked motif, demonstrating N-linkage as the common glycosylation route for M. mazei. In order to study the M. mazei N-linked octasaccharide, the glycans were chemically

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released by hydrazinolysis, after a scaled-up ConA glycoprotein enrichment. The released native glycans were reacetylated and an aliquot 1/16 was treated with O water. These glycans were then permethylated and subjected to MS/MS. De novo sequencing based on the HCD MS/MS spectra of the O and O permethylated glycans revealed a linear sequence for the glycan chain: (non-reducing end) Hex-dHex-HexNAc-dHex-Hex-Hex-Hex (reducing end). We now aim to detail the linkages and identify the monosaccharides in the glycans on M. mazei. Support: NIH P41 RR010888/ GM104603, S10 RR020946, S10 RR025082, S10 OD010724, F31 AI061886 to DRL, and DOE DE-FC-02-02ER63421.

(66) Development and Application of a Comprehensive Method for Serogroup Identification and Quantitation in Multivalent Polysaccharide-Based Meningococcal Vaccines
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The active components of most meningococcal vaccines are four antigenic serogroup polysaccharides (ACYW) derived from the bacterial capsule. Current analytical methods are complicated due to the use of multiple analytical techniques for each polysaccharide, which are also sometimes different for free or conjugated polysaccharides. An improved comprehensive method was developed, qualified and applied for the analysis of serogroup polysaccharides A, C, Y and W in a variety of meningococcal vaccines. Multivariate acid hydrolysis conditions (concentration, temperature, time) were surveyed to obtain the optimal yield of the characteristic monosaccharide from each polysaccharide repeating unit. Three hydrolysis conditions were identified (one for both serogroups Y and W, one for C, one for A). When required for the determination of Y and W, molecular size separation by centrifugal filtration removed all interfering disaccharide excipient without loss of serogroup polysaccharides. The monosaccharide products were separated and quantitated using a single customized HPAEC-PAD protocol for all four serogroup components. This serogroup polysaccharide determination method was used to analyze twelve real monovalent or multivalent polysaccharide or polysaccharide-conjugate (TT, DT or CRM197 protein) meningococcal vaccines. The products were in liquid or lyophilized powder formulations, with or without various buffers, salts and saccharide excipients. At least two, and in some cases more than 15 lots, of each vaccine were tested (>70 product lots total). Replicate operations performed by three different analysts demonstrated a method CV of <5%. The hydrolysis and chromatographic techniques were suitable for all vaccines tested. Results indicated some lot-to-lot and product-to-product variations in the contents of each polysaccharide-based antigen. However, most products were very consistent. All vaccines were within general specifications for each component, with the exception of all lots of one polysaccharide vaccine – which were found to be deficient in the serogroup A component only. The deformation, acid hydrolysis and chromatographic techniques were demonstrated to be highly reproducible and robust, exhibiting an increase in precision, level of accuracy and efficiency compared to current methods. These techniques may be adaptable for the evaluation of other types of polysaccharide-based vaccines. We have used this method in several international collaborative studies with other regulators and standards organizations.

(67) Studies on the extraction of polysaccharide from oyster Crassostrea gigas and its activities on anti-oxidation in vivo
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The fresh oyster (Crassostrea gigas) was then separated as the precipitant from 70% of ethanol. The yield and the polysaccharide content was 18.95% and 81.23% respectively. Sixty mice were randomly assigned into 6 groups of ten each, which were physiologic saline, physiological saline without 50% ethanol by intragastric administration (ethanol negative), Bifendate (200mg/Kg·d), and oyster polysaccharide of low, medium and high doses of 80, 160, 320 mg/Kg·d respectively. The mice were treated with the agents each day for four weeks and then after 50% ethanol was administrated intragastrically. The results illustrated that for the model control group, the activity of the serum ALAST went up to 31.12 U/L and 37.74 U/L from 8.18 and 11.52 U/L respectively, while the values for those of polysaccharide groups with all the doses were significantly decreased, in the range of 7.79-8.82 U/L and 12.34-13.91 U/L respectively. Comparing with the findings determined for model control, control and Bifendate groups, ADH activities of polysaccharide groups significantly decreased when compared with the data for model control group. As the product of lipid oxidation, the content of MDA in mice tissue decreased significantly for the mice administrated with polysaccharide of all doses and, the data obtained for the low and high doses groups was even better than that of Bifendate group. SOD activity represented anti-oxidation potential of the liver tissue and the results obtained for the mice groups administrated with medium and high doses of oyster polysaccharide increased significantly. Therefore, oyster polysaccharide could be potential anti-oxidation agent for food additive or pharmacy uses.

(68) Heavy metals removal of modified chitosan from scallop processing waste hydrolyzate
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Three kinds of modified chitosan including salicylaldehyde/epichlorohydrin modified chitosan, chitosan loaded on Silicagel and nano-silica/chitosan (DNS-CTS) were synthesized by modified method with chitosan and other materials in this study. The effects of each modified chitosan to remove heavy metals were tested by using Cd solution. The results showed that the removal effect of nano-silica/chitosan (DNS-CTS) was the best. So DNS-CTS was
used in the further study. Static adsorption experiments were performed to the heavy metals Cd, Pb and Cr in the hydrolysis of scallop processing waste with DNS-CTS to study the impacts of dosage, initial pH and removal time on absorption properties of DNS-CTS. The results showed that DNS-CTS had strong ability to remove heavy metals in the hydrolysis of scallop processing waste. When conditions reached 0.005g/ml DNS-CTS addition, pH 6 and adsorption time of 180min, the removal effect would be better. Under these conditions, the removal rate for the heavy metal of Cd, Pb and Cr could reach 81.36%, 62.83% and 74.99%, respectively. What’s more, the retention rate of amino nitrogen of scallop processing waste hydrolyzate was 98.96%. It indicated that DNS-CTS had little effect on the content of amino nitrogen. Modified chitosan materials DNS-CTS can be used to remove heavy metals from scallop processing waste hydrolyzate. This work was supported by Programme of Oceanic and Fishery Department of Liaoning Province (201004).

(69) Interaction of the yeast Klus killer toxin with the cell wall of susceptible Saccharomyces cerevisiae strains

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The yeast killer phenomenon was first described in the early 1960s and has been extensively studied ever since. The mechanism of action of the “classic” S. cerevisiae toxins K1, K2 and K28 is well known. All three have a common first step, energy-independent, in which they adsorb to a receptor in the cell wall of the sensitive strain. Toxins K1 and K2 bind to beta-glucan, while K28 recognizes a mannanprotein. Just two years ago a new toxin, Klus, was described, but its mechanism of action is still unknown. In order to investigate the nature of the cell-wall receptor for the Klus toxin, we conducted killer tests consisting of inoculating methylene blue YEPD plates with patches of yeasts of known killer phenotype. The plates were previously seeded with a lawn of a sensitive strain. The growth inhibition halos of the patches on the lawn strain were measured, when present. We used all known S. cerevisiae killer types for the patches: K-, K1, K2, K28 and Klus. As lawn strains, we used wild type S. cerevisiae and a collection of cell-wall defective mutants, including mnn, ktr and kve. The experiments were done at two pHs (3.2 and 4) and two temperatures (12 and 20 °C). In agreement with previous findings, the results indicate that K1 and K2 toxins interact with beta-glucan, and K28 interacts with mannanproteins, being the terminal alpha 1,3 mannoses required for the receptor. In addition, we found that strains with defects in beta-glucan biosynthesis, kve1, kve3 and kve11, showed an unexpected resistance to the K28 toxin. These results suggest that the integrity of the beta-glucan layer is also important for the “in vivo” interaction of K28 with its receptor. The Klus strains killed all analyzed lawns except those just mentioned with defects in beta-glucan biosynthesis. In addition, the mutants with the most drastic defects in mannanprotein biosynthesis (i.e. mnn9) showed an increased sensitivity to the Klus toxin. This suggests a direct interaction of Klus with the beta-glucan layer of the cell wall, which can be facilitated in strains in which the external mannanprotein layer is damaged.

(70) Construction of cassettes for deletion of the repressors of glycosyl hydrolase enzymes in Trichoderma reesei by fusion PCR

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Cellulases and hemicellulases were glycosyl hydrolases that act hydrolyzing the β-1,4 linkages of cellulose and hemicellulose, and have great interest in biofuel industry. So, glycosyl hydrolase enzymes produced by Trichoderma reesei mutant strains has been attracting interest in the industry sector. This microorganism has two repressors which are involved in cellulases transcriptional regulation. The deletion of both repressors was not performed in the same strain. The aims of this work were constructing two deletion cassettes by fusion PCR to deletion of regulation genes in T. reesei. The molecular markers were pyr4 (uridine auxotrophy) and hph (hygromycin B resistance) genes to deletion cassette 1 and 2, respectively. First, the DNA of T. reesei was extracted and used to amplify the pyr4 gene by PCR, followed by amplification of the promoter and terminator regions of target genes 1 and 2. After that, was performed the fusion PCR with promoter and terminator regions of target gene 1, and pyr4 molecular marker, intending to make the deletion cassette 1. Furthermore, was performed other fusion PCR with promoter and terminator regions of target gene 2, and hph molecular marker, intending to make the deletion cassette 2. After, the products obtained of these PCRs were used to perform the last amplification to obtain the final deletion cassettes. All reactions were performed using High Fidelity Taq Polymerase. The electrophoresis show an expected band at 5418 pb and 3525 pb corresponding to deletion cassettes by fusion PCR to deletion of regulation genes in T. reesei. The next step will be to use these deletion cassettes to transform T. reesei to obtain mutant strains with high secretion of cellulases and hemicellulases. Besides, these results may contribute to better understand of the regulatory mechanisms and functional properties of glycosyl hydrolases from T. reesei.

(71) Conserved ion and amino acid transporters identified as phosphorylcholine modified N-glycoproteins by metabolic labeling with propargylcholine in Caenorhabditis elegans

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Parasitic nematodes synthesize Pc-modified biomolecules that can modulate the host's antibody and cytokine production to favor nematode survival, contributing to long-term infections. Only two nematode Pc-modified proteins have been unequivocally identified, yet discovering the protein targets of Pc modification will be paramount to understanding the role(s) that this epitope plays in nematode biology. A major block in the field has been the lack of techniques for selective purification of Pc-modified proteins. The non-parasitic nematode Caenorhabditis elegans expresses Pc-modified N-linked glycans, offering an attractive model to study the biology of Pc-modification.
We developed a robust method to identify Pc-modified proteins by metabolic labeling of primary embryonic C. elegans cells with propargylcholine, an alkyne-modified choline analog. Cu(I)-catalyzed cycloaddition with biotin-azide enables streptavidin purification and subsequent high-throughput liquid chromatography and mass spectrometry identification of propargyl-labeled proteins. Peptides resulting from trypsin digestion and peptides from PNGase F digestion were analyzed by LC-MS/MS. PNGase F releases most oligosaccharides from N-linked glycoproteins, deamidating the modified Asn to Asp and resulting in a +0.98 Dalton mass shift at that residue. Thus, sites of PG-choline-N-glycan attachment were identified by including this mass shift as a variable modification for MS analysis. All proteins identified using stringent criteria are known or predicted to be membrane or secreted proteins, consistent with the model of a Golgi-resident, putative Pc-transporter. Of the 57 Pc-N-glycosylation sites reported, 35 have been previously observed as N-glycosylation sites in high-throughput screens of C. elegans. Several identified Pc-proteins are nematode-specific proteins, but nine of the Pc-modified proteins are widely conserved ion transporters and amino acid transporters, while twelve others are conserved proteins involved in synaptic function. This suggests functional role for Pc-modification beyond immunomodulation. This work supports the amenability of propargylcholine metabolic labeling as a robust method for the identification of Pc-modified N-glycoproteins in C. elegans and related nematodes to further our understanding of the pathophysiological role of Pc. Interestingly, the incidence of auto-immune and inflammatory diseases, such as type I diabetes and multiple sclerosis, are reduced in areas of endemic nematode infection. Thus, Pc-proteins are being investigated for their therapeutic potential in treating inflammatory and auto-immune disorders.

(72) Analysis of N-glycoproteins using Genomic N-glycosite Prediction
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Protein glycosylation has long been recognized as one of the most common post-translational modifications. Most membrane proteins and extracellular proteins are N-linked glycosylated and they account for the majority of current clinical diagnostic markers or therapeutic targets. Quantitative proteomic analysis of detectable N-linked glycoproteins from cells or tissues using mass spectrometry has potential to provide biological basis for disease development and identify disease associated glycoproteins. Here, we show the feasibility of N-glycopeptide identification and quantification using genomic N-glycosite prediction (GenoGlyco) coupling with stable isotopic labeling and accurate mass matching. The accurate mass detection of isotypically labeled peptides by mass spectrometry allowed assigning detected peak pairs to N-linked glycopeptides predicted from genes expressed in the cells. The method was applied to the analysis of N-glycoproteins from two ovarian tumor cell lines with stable-isotope labeling by amino acids in cell culture (SILAC) to identify all possibly detectable N-glycopeptides. We then applied the stable isotope-labeled glycopeptides from the two cell lines to the identification of N-glycopeptides from ovarian tumor. We showed that the described method has great potential in analysis of detectable N-glycoproteins from cells and tissues.

(73) Specific glycopeptides from serum: a novel class of biomarkers
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Over the last decades, several successful studies have emerged on the detection of candidate glycan biomarkers for several disease including auto-immune diseases and multiple types of cancer. So far, most studies have focused on whole biofluids and it seems likely that changes in specific glycoforms of proteins will result in clinically more important biomarkers, with improved sensitivity and specificity. Triple quadrupole mass spectrometry allows for the sensitive and specific analysis of molecular ions, and is therefore an excellent tool for the development of such markers. Here, we present methods to investigate protein-and site-specific glycan profiles of the immunoglobulins A, G and M that are associated with inflammatory diseases and cancer. First, the glycosylation pattern of the protein standards was determined using trypic as well as pronase digestion. Both digests were profiled using an nLC-Chip/QTOF MS and glycopeptides were identified using GFPinder, an in-house-software tool. The identified glycoforms were then quantified using multiple reaction monitoring of the tryptic glycopeptides using UPLC-QqQ MS. Using this method, 26 glycopeptides were monitored for IgG, 20 glycopeptides for IgA and 17 for IgM, all with high accuracy and low limits of detection. First, the method was applied to serum samples from Epithelial ovarian cancer patients (n = 40) and their age-matched controls (n = 40). Eighteen glycoforms were shown to alter with OC, and using LOOCV, the accuracy of prediction was shown to be 92.5%, with a specificity of 97.5% and a sensitivity of 87.2%. These findings were later corroborated in a second set of serum samples from EOC patients, further validating these results. Immunoglobulins are known to play an important role in inflammatory diseases, and therefore, we applied our method to a set of patients with primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC). Significant differences were observed between the disease groups and controls for glycopeptides from all proteins: IgG, IgA and IgM. Data will also be shown from samples from lung cancer patients, HIV-infected individuals and patients with scleroderma. We here show, for the first time, the large potential of triple quadrupole mass spectrometry in the development of highly specific glycopeptide markers for multiple diseases.
(74) High-Throughput Human Serum N-Glycan Profiling with GlycoGrid 4D Visualization and Automated Annotation Using GlyQ-IQ Multi-Core Software and PNNL Institutional Computing
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N-Glycan profiling has been gaining in popularity for disease biomarker discovery. The composition and structures of glycans released from human serum and plasma have led to the detection of several candidate biomarkers that differ between disease and healthy state across a wide range of cancers. Several recent publications have presented glycan compositions and structures that are significantly changing in abundance in cancer disease state relative to control. Glycan profiling has also led to novel glycoforms observed on glycoproteins (epidermal growth factor receptor) excreted from cancer cell lines. Global N-glycan profiling can generally be categorized into two general approaches: chemically derivatized and non-derivatized. Our approach is considered non-derivatized and involves minimal sample preparation to decrease sample handling and help maintain the native form of the glycan and preserve the natural distribution of glycoforms present. The method involves protein denaturing, microwave assisted PNGase F N-glycan release, sodium borohydride reduction, and automated graphitize carbon purification. This method is designed to establish a 1-to-1 correspondence between a single LC-MS feature and a glycan structure with isomer resolution. Glycomics Quadra variant Informed Quantification (GlyQ-IQ) is a targeted software suite (written in C#) designed for annotating and quantifying N-glycan LC-MS datasets. Expanded retrosynthetic glycan libraries are used to populate the glycan targets of interest and each target is extracted from the data. Extraction is based on quadravariant detection which includes isotope profiles, exact mass, LC peak shape, and glycan relationship information. Using multiple orthogonal pieces of information increases the confidence of the assignments. Since each target is discrete, they can be executed in a parallel across multiple cores in a HPC environment. Our C# applications were run on Windows 2008 High Performance Compute cluster running on AMD Interlagos chips (2.2GHz) and with 32 cores per node (2 socket x 16 core). Run times were decreased by over 95% from single core operation. The glycan profiles generated are viewed with the GlycoGrid 4D viewer that displays 4 dimensional monosaccharide compositional data in a 2 dimensional format. Plotting the glycan compositions in this format visualizes glycan families and clusters of related glycans.

(75) Towards identifying the O-fucose proteome
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O-linked fucosylation is known to take place at distinct consensus sequences on properly folded Epidermal Growth Factor-like (EGF) repeats and Thrombospondin Type-1 repeats (TSRs) by the action of protein O-Fucosyltransferases 1 (POFUT1) and 2 (POFUT2) respectively. Elimination of Pofut1 in mice results in embryonic lethality with a Notch-like phenotype, emphasizing the role of O-fucosylation of Notch in its function, while elimination of Pofut2 results in an embryonic lethal phenotype although the specific mechanism is unknown. To better understand the biological function of O-fucosylation, we need to identify all targets of POFUT1 and POFUT2. Database searches with the consensus sequences predict >100 targets for POFUT1 and over 50 for POFUT2, but recent papers have speculated that O-fucosylation may occur in other settings as well. Thus, we are developing an unbiased proteomics strategy to identify all O-fucosylated proteins, or the O-fucose proteome. We have previously shown that the sugar analog 6-alkynyl fucose (6AF, also known as 5-alkynyl fucose) is a bio-orthogonal analog of fucose, in so far as it is utilized by POFUT1 and POFUT2 and efficiently incorporated into the appropriate consensus sites in EGF repeats and TSRs and is elongated by Fringe or β3-GlCl as appropriate on EGF repeats and TSRs, respectively. Our protocol for identifying the O-fucosyl proteome in a target system involves collecting cell lysates and media fractions from cells cultured in the presence of 6AF, subjecting them to Cu(I) catalyzed azide-alkyne cycloaddition ("click reaction") with azido-biotin, pulling down labeled proteins with streptavidin beads, and subjecting them to on-bead protease digestion. The peptides are analyzed by nanoLC-MS/MS and compared to the GPM database to identify isolated proteins. Our preliminary results indicate that we can selectively isolate overexpressed proteins with EGF repeats or TSRs, such as mouse Notch 1 EGF 1-5 and human Thrombospondin-1 TSR 1-3. Our continuing work is to refine our methods and increase the scale of protein examined, in order to identify endogenously O-fucosylated proteins. Additionally, having access to Pofut1−/− and Pofut2−/− cells will enable the identification of the specific proteins which are O-fucosylated by each fucosyltransferase. This work was supported by NIH grants GM061126 and CA12307101.

(76) N-link Glycan Serves as a Determinant in Microvesicle Cargo Recruitment
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Microvesicles are nano-sized membrane particles secreted from nearly all mammalian cell types. Much evidence has revealed that microvesicles have critical roles in many physiological process including cell-cell signaling, immune activation and suppression. They are also closely related to many disease and tumor progression. The biofunctions that microvesicle carries are much dependent on its cargo components, the regulation of which, however, is little known. A few microvesicle cargo recruitment mechanisms have been proposed, but little attention are paid on N-link glycosylation of microvesicles. Earlier studies in our lab indicated that HIV, which had similar structure and topology with microvesicle, originated...
from cell membrane microdomains that had distinct glycan signatures. The following study further revealed that microvesicles released from human cancer cell lines had a glycomic pattern that featured in high mannose, poly-LacNAc and complex, and the feature was very different from that of parent membrane. It suggested a potential biogenesis pathway of microvesicles that mediated by glycosylation. In this study, we characterized a MV cargo glycoprotein EWI-2 that had N-link glycan dependent recruiting pathway. Removal of carbohydrate attached on the protein severely disrupted its incorporation within MV. Besides, the overall alteration of glycose change the recruitment of a few MV-enriched glycoprotein and its associated proteins. Taken together, our data provided evidence that N-link glycan directs glycoprotein sorting in MV and serves as a determinant of MV cargo selection signal.

(77) Aminated Human Milk Fucooligosaccharide Library and Corresponding High Density Neoglycoproteins
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Using ammonium carbamate, 12 main individual fucooligosaccharides (FOS) from human milk (2-FL, 3-FL, DFL, LNFP I, II, and III, LND I, MFLNH III, DFLNH a, DFLNnh, TFLNH, and MF (1-3)ILNO were converted into the corresponding glycosylamines. Acylation with N-hydroxysuccinimide ester of N-t-BOC-glycine gave, after deprotection, FOS-Gly derivatives with a free amino group on a multimilligram scale with 70% yields. The same modification of the total pool of delactosylated neutral human milk OS gave rise to a mixture of the corresponding N-t-BOC-Gly-OS. After HPLC and deprotection, a library of 25 individual FOS-Gly derivatives was obtained. These included, together with 12 main FOS: LND II, LNnd I, MFLNH I and II, MFLNhH I and II, MFpLNH IV, DFLNH b and c, DFpLNH II, DF(1-2,1-3)iLNO, TF(1-2,1-2,1-3) iLNO, and PFiLNO. Five Gly-OS core-based derivatives, including lactose, LNT, LNnt, LNh, and LNnh, were also prepared. By carbodiimide chemistry, BSA was derivitized with ethylenediamine, and succinyolated with the corresponding anhydride to give succ-BSA. Using carbodiimide coupling, 10 suc-BSA-FOSn neoglycoproteins with a high oligosaccharide substitution (n > 40) were prepared. Interaction of suc-BSA-FOSn with fucoolectins: Laburnum anagyroides bark lectin (LABA), perch (Perca fluviatilis) oocyte lectin (PFL), sander (Lucioperca lucioperca) oocyte lectin (LLL), and Lotus tetragonolobus agglutinin (LTA) was studied. Another application of FOS-Gly includes glycoarrays and glycochips preparation.

References


(78) Enhanced Mannosyl-phosphorylation in Glyco-engineered Yeast by Heterologous Expression of YIMPO1
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Some portions of N-glycans are phosphorylated in yeast by the dual action of two proteins encoded by MNN4 and MNN6. Mnn4 protein (Mnn4p) has been known to be a positive regulator of Mnn6p, a real mannosyltransferase adding mannosyl-phosphate residues to glycan. In the present study, YIMPO1, Yarrowia lypoldtica homologue of MNN4, was used to increase the mannosyl-phosphorylation of N-Glycans in Saccharomyces cerevisiae without the help of Mnn4p or Mnn6p. N-glycan analysis of yeast cell wall mannoproteins clearly showed that the YIMPO1 expression greatly increases the amount of mannosyl-phosphorylated glycans in ScMNN4 and ScMNN6 double deletion mutant as well as wild-type. Furthermore, increased amounts of mannosyl-phosphorylated glycans were much higher upon the heterologous expression of YIMPO1 compared to recombinant overexpression of ScMNN4. This strategy employing recombinant expression of YIMPO1 was applied to the glyco-engineered S. cerevisiae strain in which yeast-specific glycosylation pathway was abolished. The resulting glycans containing highly increased amount of mannosyl-phosphates were converted to mammalian-type N-glycans with mannose-6-phosphate by uncapping the outer mannose residue. This glyco-engineered yeast will have the promise for the production of therapeutic enzymes containing a high content of mannose-6-phosphates for lysosomal storage diseases.

(79) Investigation of the Factors That Affect Glucose Unit Generation in HILIC-based Analyses
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Analysis of such complex structures is typically achieved using a HILIC-based approach, which allows for separation of both neutral and charged glycan structures within a single chromatographic window. In an effort to standardize glycan separation data for curation in databases, glycan peaks are often converted to glucose unit values, which aid in normalizing variability typically observed between instruments and labs. However, adjustment to chromatographic conditions within the separation method can affect the glucose unit values, therein leading to the possibility of erroneous glycan assignments. Here, we present a study of the impact of modifying a number of chromatographic parameters for N-linked glycan separation. Factors such as temperature, gradient slope, mobile
phase ionic strength, flow rate and sorbent particle size have been evaluated with regard to their effect on glucose unit values. The data presented provides a guide to N-glycan method optimization where the end user requires glucose units for their analytical workflow.

(A) A new fluorescent-labeled monofluorinated cyclooctyne for imaging cell-surface glycoprotein expression in metastatic melanoma

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Aberrant glycoprotein expression is increasingly implicated in the development and progression of human disease, including melanoma. Imaging of cell-surface glycosylation has emerged as a powerful tool for developing an understanding of this phenomenon. Techniques that have been employed frequently rely on so-called "click" chemistry approaches that afford supreme site-selective conjugation of fluorescent labeled constructs to glycoprotein epitopes (primarily azide-modified sialic acids and sugars). Our work in the development of site-selective click reagents led us to explore the potential of a monofluorinated-cyclooctyne moiety (MFCO), synthesized in our laboratory for other bioconjugate chemistry applications, as a potential alternative to current commercially-available click-chemistry reagents used for this application. Within this context, we functionalized the MFCO with a fluorescent label and compared the reactivity of our new copper-free click chemistry reagent (MFCO-FITC) to commercially-available AlexaFluor 488 DIBO-alkyne. The comparison was undertaken by incubating malignant melanoma and non-malignant melanocytes with azide-modified tetraacetylated sugars (azido-galactosamine; azidomannosamine; and azido-glucosamine) in standard media for up to three days according to methods described previously. Following the incubation period, cells were washed and incubated for up to one hour with the DIBO-alkyne and the MFCO-FITC to promote the click reaction of the fluorescent probes to the azide-modified sugar moieties expressed by cell-surface glycoproteins. Following the click reaction, cells were imaged by standard microscopy techniques and the resulting images were compared. Our results demonstrate that the MFCO-FITC fluorinated-cyclooctyne group is sufficiently stable to undergo the site-selective click reaction with azide-modified sugar epitopes on the surface of malignant and non-malignant cells. Further, we found no significant difference between the performance of the MFCO-FITC in comparison to the commercially-available DIBO-alkyne. Our data further demonstrate that differences in the uptake and cell-surface expression of sugar moieties can be identified by the use of the MFCO-FITC click-chemistry approach. These findings suggest the MFCO-FITC is a promising alternative to the DIBO for imaging of cell-surface glycosylation. Further studies are required to examine the use of the new MFCO-FITC moiety for more refined in vitro and in vivo applications.

(80) SimianTools: A software suite for enhancing throughput and evaluation of mass spectrometry-based glycomics datasets

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Advances in the sensitivity and robustness of mass spectrometry (MS) have enabled comprehensive analysis of glycans in complex mixtures by overcoming the limitations imposed by the small sample amounts usually available from starting materials of high biomedical interest. However, MS and MSn analyses create immense volumes of data, which generally require significant manual interpretation. Complete evaluation of these datasets is a major bottleneck for glycomic high-throughput. Although software applications such as the freely available GlycoWorkbench or the commercial program SimGlycan® are available, each has limitations, especially in regards to the analysis of complex samples. GlycoWorkbench provides valuable visual representations and simulation of glycan fragmentation, but does not support easy processing of hundreds of spectra at once. SimGlycan® is capable of interpreting thousands of spectra in a high-throughput fashion, making use of MS/MS fragmentation data. However, the database used by SimGlycan® relies on minimally-or non-curated public databases that contain duplications, errors, and oversights, resulting in low quality annotation of MS data. In addition, SimGlycan®’s post analysis output provides very little content useful for subsequent interpretation. To help fill the void that currently exists in effective automated glycomics interpretation, we have developed several tools that, in combination with existing tools, improve upon SimGlycan® results. First, we developed a web based curation (Qrator) environment and a program called SimglycanDatabaseBot allowing users to create customized databases of N-glycan, O-glycan, and glycosphingolipid structures, thereby improving the annotation of MS-based annotation by removing erroneous structures and reducing redundancies in existing databases. Second, we developed Simian Tools to enhance the evaluation of SimGlycan® annotation results. Simian Tools utilize the functionalities of GlycoWorkbench to generate graphical representations of the glycans assigned by SimGlycan® and also add statistical measures, structural topology features, and quantitative information to assist users in the evaluation of the total dataset. Not only is it possible to visualize the results of a single experiment, but our program also facilitates easy comparison of multiple experiments.

(81) The GlycomeAtlas tool to aid in the visualization of glycan structure expression in human and mouse

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Many technologies such as tandem mass spectrometry and high-performance liquid chromatography can be used to obtain the glycan structure profile of biological samples. Moreover, the Consortium for Functional Glycomics (CFG) and UniCarbKB have
made such data publicly available on the Web. In order to aid researchers in analyzing such data, we present the newest addition to RINGS: the GlycomeAtlas tool (http://www.rings.t.soka.ac.jp), which is a visualization tool that displays the glycan structures that have been profiled in a particular tissue in human or mouse. In this tool, there is a sketch of human and mouse and their organs. An organ can be individually clicked, upon which the list of glycan structures that have been profiled in the selected organ’s tissue sample is displayed. Moreover, individual glycans in the displayed list can be clicked as well, upon which the tissues in which the selected glycan have been found are highlighted. GlycomeAtlas also provides search functionality to search for a particular glycan structure across all samples, and the resulting tissues are highlighted on the display. This tool also allows for the user’s local profiling data to be visualized by taking profile data from a text file and displaying the contents. The GlycomeAtlas tool was originally released using the human and mouse profiling data from the CFG. Recently, we have also added human serum data that was profiled by HPLC, based on a collaboration with Niigata University. Therefore, it is possible to add new data to GlycomeAtlas as they are published.

(83) AWeb tool for visualizing common patterns among glycans
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Our research aim is to predict glycan recognition patterns for the purpose of the elucidation of glycan recognition mechanisms. Due to the advancement of glycomics technologies, many experimental results are being accumulated from mass spectrometry data, microarray data, glycan array data, etc. Information of the experimental data related to glycans and have been published in databases that integrate glycan information such as KEGG GLYCAN, the Consortium for Functional Glycomics (CFG), Glycosciences.de and JCGGDB. Because glycan structures have a complex structure, and since tools that can analyze these experimental data are few, manual analysis is very time-consuming. Therefore, we have developed a web tool called MCAW (Multiple Carbohydrate Alignment with Weights) for the extraction of binding recognition patterns from glycan array data. The MCAW algorithm is based on the KCaM algorithm, which aligns pairs of carbohydrate structures, and ClustalW, which is a popular multiple amino acid sequence alignment algorithm. The MCAW tool has now been implemented on the web as a part of RINGS to output a multiple glycan alignment of a set of glycan structures. We hope that this tool can aid in the analysis of lectin recognition patterns which can be analyzed using data sets of glycan structures from binding affinity data. Many of such data can be obtained from glycan array data such as that from the CFG. Additionally, it is possible to configure parameters in the calculation of the alignment such that monosaccharides and bonds are aligned appropriately for the data set. Presently in the MCAW tool, we have confirmed the possibility of aligning up to 88 glycans at once, and we will present some examples of alignments obtained from CFG data.

(84) Semantic Technologies for Glycoinformatics
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Glycoinformatics is an emerging discipline that attempts to integrate diverse information, including the structures and abundances of glycans and glycoconjugates in various biological samples, the attachment sites and site occupancy of glycans in glycoconjugates, the transcription levels for numerous glycogenes (which encode glycan-related proteins such as glycosyltransferases), the abundance of carbohydrate active enzymes, the activity of these enzymes and the biological consequences of perturbing these factors in vivo. Due to the complexity of this endeavor, new computational methods must be developed to supplement the more traditional informatics methods currently used to discover knowledge in genomics and proteomics datasets. Our approach to this challenge is to develop semantic technologies (including ontologies) to represent information in the glycomics domain and then use this information as the basis for data annotation and knowledge discovery. The semantic infrastructure we have developed and its use in software applications for information processing and knowledge discovery are described.

(85) Functional Glycan Epitopes: Sequential Mass Spectrometry and Library Matching
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Higher stage sequential mass spectrometry in the ion trap affords the ability to isolate and dissociate oligosaccharide substructures, enabling the determination of structural detail in complex samples. Certain substructures (epitopes) are responsible for multitudes of biological activity, largely through interaction with glycan binding proteins. Typically, with oligosaccharides released and permethylated, these resident epitopes can be isolated and dissociated in the ion trap. A complication in the analysis of such structures is that these epitopes are frequently isomeric. However, the collision-induced dissociation of these substructures can produce unique mass spectral patterns which can be used to unambiguously assign structural details. A representative set of oligosaccharide standards was obtained through commercial and academic sources. Subclasses were sequentially isolated via solvent extraction (glycosphinogolipids), enzymatic N-glycan release, and chemical O-glycan release. Permethylated samples were directly infused into an ion-trap mass spectrometer equipped with a nanoelectrospray source.
Spectra obtained from various standards were used for spectral matching purposes and data from various biological materials were queried against this library of standards. Spectral comparisons indicated distinct differences, both in terms of fragment masses and intensity patterns, between isomeric fragments. Replicate acquisitions were obtained, from multiple standard materials where possible, to assess spectral reproducibility. This analysis was most useful and interesting for the various fucosylated lactosamine structures: H1 antigen, H2 antigen, Lewis A, and Lewis X, where distinct mass spectral differences were observed. In general, spectral reproducibility was very good, even when compared to similar substructures from different precursors. This held true even over long periods of time (months and years) between analyses. Achieving suitable spectral quality was of paramount importance, and was achieved through signal averaging. Epitope spectra were from various sources, including glycosphingolipids, N-linked, and O-linked glycoprotein glycans. Various fucosylated lactosamine spectra were compared to corresponding standards to detail specific substructures found, including isomeric mixtures.

(86) MALDI Imaging Mass Spectrometry of N-Glycans in Frozen and FFPE Tissues
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A new Matrix Assisted Laser Desorption Ionization Imaging Mass Spectrometry (MALDI-IMS) method to spatially profile the location and distribution of multiple N-linked glycan species in frozen or formalin-fixed tissues is described. The method allows the simultaneous profiling of multiple glycan species released from intracellular organelle and cell surface glycoproteins, while maintaining histopathology compatible preparation workflows. A recombinant peptide N-glycanase F (PNGaseF) enzyme was sprayed uniformly across mouse tissue slides, incubated for two hours, then sprayed with DHB or CHCA matrix for MALDI-IMS analysis on a Bruker Solarix MALDI FT-ICR mass spectrometer. An antigen retrieval step was added for formalin-fixed tissues. Using this basic approach, global snapshots of major cellular N-linked glycoforms were detected, including their tissue localization and distribution, structure, and relative abundance. Depending on the tissue and preparation, approximately 40 glycan species can be readily detected. Off-tissue extraction and modification of glycans from similarly processed tissues and further mass spectrometry or HPLC analysis was done to assign structural designations. Co-treatment of tissues with PNGaseF and sialidase can be used to detect larger glycan structures, as well as aid in the structural assignments. Novel renal cell carcinoma and non-small cell lung carcinoma tissue slices that contained both non-tumor and tumor regions were assessed with the PNGaseF and glycan imaging workflow. Multiple glycan species are detected that are specific to either tumor or non-tumor regions in both cancer tissue types. From a clinical diagnostics perspective, the ability to differentially profile N glycans and correlate their molecular expression to histopathological changes can offer new approaches to identifying novel disease related targets for biomarker and therapeutic applications.

(87) Identification and Characterisation of Lewis Antigens on Ovarian Cancer N-glycans using MALDI-QIT-TOF
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Currently, there are no reliable biomarkers for ovarian cancer, and most diagnoses are late stage, resulting in a 56% average mortality rate within 5 years. The Lewis blood group antigens are terminally fucosylated LacNAc structures on N- and O-glycans, and they have been studied as potential biomarkers for ovarian cancer (Escrevente et al., 2006). The exact type of Lewis antigen is critical for diagnostic value, and attempts at detecting and characterising Lewis antigens on ovarian cancer glycans have been hindered by a lack of antibodies, lectins and fucosidases that can reliably discriminate between all the types of Lewis antigens. Tandem Mass Spectrometry is a reliable method for characterising the terminal Lewis antigens. Upon ionisation and fragmentation of cellular glycans, terminal Lewis antigens can be identified based on unique masses, fragmentation patterns and diagnostic ions. In this work, a Shimadzu MALDI-Quadropole Ion Trap-TOF capable of up to four tandem fragmentations (MS5) was used to characterise Lewis antigens on N-glycans from an ovarian cancer cell line. The N-glycans were enzymatically released from an ovarian cancer cell line, permethylated and purified using reversed-phase chromatography. Not only were we able to confirm previously undetected N-glycans carrying terminal fucose, but using the MS5, we were also able to unambiguously identify terminal Lewis X on several of these N-glycans. Therefore, our results raise the possibility that Lewis X could be a biomarker candidate in ovarian cancer. In addition, by being able to fully characterise terminal Lewis antigens on cancer cell glycans, we demonstrate a powerful approach in the hunt for cancer biomarkers.

Reference

(88) Profiling IgG Asparagine-Linked Oligosaccharides by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection
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Thermo Fisher Scientific

Antibodies are among the largest class of glycoproteins studied as potential therapeutic proteins with over 30 monoclonal
antibodies (mAbs) approved by the U.S. FDA. Development of mAbs, antibody-drug conjugates, and multi-functional antibodies is an active area of research. Understanding and characterizing product glycosylation as a potential critical quality attribute is of importance with evidence that glycosylation may impact biological efficacy, pharmacokinetics, and cellular toxicity. Protein expression systems and reactor conditions can impact glycosylation, leading to potentially undesirable glycosylation. For example, high-mannose species may be present, which are not typical of human antibody glycosylation. The presence of these species in the Fc domain has been linked to increased serum clearance of IgG antibodies. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is an effective tool for analyzing glycans present in glycoprotein therapeutics. In this work, a method for analyzing the asparagine-linked -glycans commonly found on antibodies is described. Initial identification and mapping is performed by comparison to known standards in conjunction with selective exoglycosidase digestion of both standards and glycans released from human IgG. Both neutral and charged glycans are identified in a single injection, but the method’s emphasis is on resolution of the neutral N-linked oligosaccharides, which are typically 90% of the IgG’s N-linked oligosaccharides. The neutral glycans are well resolved with separation of common high-mannose species from typical human IgG-type glycans, including the resolution of the high-mannose oligosaccharide containing five mannose residues (Man5) from the complex biantennary oligosaccharide containing no galactose residues (G0). The HPAE-PAD retentions of known glycans are indexed to the retention of maltose and a series of maltodextrin standards to define an index value characteristic of the glycan. Method ruggedness was evaluated across systems, analysts, and column lots with retention time precision RSDs between 0.63 and 4.0% and retention indices precision RSDs between 0.27 and 1.1%. These retention indices were used to aid in identification of glycans released from example monoclonal antibody samples of unknown glycosylation.

(89) N-Glycan Analysis of IgG by Enzymatic Digestion with Remove-iT Endo S and PNGase F Glycerol Free
Elizabeth McLeod, Colleen McClung, Alicia Bielik, Paula Magnelli, Nathan VerBerkmoes, Ellen Guthrie
New England Biolabs, Inc

Biotechnology companies are currently developing a growing number of monoclonal IgG antibodies as therapeutic agents. Critical for the structure and biological activity of the molecule is the N-glycan moiety attached to the asparagine 297 residue in the constant domain of the antibody. There are many variables in cell culture systems that can greatly influence the heterogeneity of the glycans on IgG. For this reason it has become increasingly important to monitor the glycosylation profiles of these biotherapeutics in the production process. Remove-iT Endo S (also known as Endoglycosidase S) has a high specificity for removing the N-glycan moiety of IgG. It was cloned from Streptococcus pyogenes and overexpressed in E. coli as a fusion to the chitin-binding domain. IgG samples were enzymatically deglycosylated under native conditions using Remove-iT Endo S and PNGase F Glycerol Free. The deglycosylated IgG samples were then analyzed by SDS-PAGE gel shift analysis to estimate the degree of deglycosylation. In addition, nanoLC-TOF MS was used to analyze the glycans released from IgG following deglycosylation by both enzymes. Remove-iT Endo S cleaves the biantennary N-glycan moiety of IgG after the first N-acetylgalcosamine (GlcNAc) residue, leaving only the GlcNAc with or without a core fucose residue. However, PNGase F cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins. Remove-iT Endo S is a more robust enzyme for the method described herein as it completely removes the sugar residues from IgG. Conversely, the PNGase F glycerol free digest does not result in a complete digestion under native conditions.

(90) Structural characterization of glycans and glycoproteins: redefined glycosidases for an enhanced orthogonal approach
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New England Biolabs, Inc

Because their biosynthetic route is not template-driven, glycans display a high structural diversity. Detailed characterization often requires an orthogonal approach combining liquid chromatography, mass spectrometry, and/or lectin affinity binding. However, to confirm or define some structural features (anomeric configuration, isomer identity) these techniques are often complemented by enzymatic sequencing. Well-characterized exoglycosidases are key to accurate sequencing of glycans. The ideal exoglycosidase has high activity and tight specificity, which is defined as the ability to extensively cleave a given residue from a glycoconjugate, without removing any other. Unfortunately many broad specificity exoglycosidases often have preferential rates for some kinds of linkages. Another feature, which must be taken into account, is the enzyme’s different catalytic rates depending on whether the residues are linear or branched. Additionally, we have observed that some enzymes fail to act on glycoproteins or glycopeptides, while still fully active on an isolated glycan molecule. We present here a revised characterization of glycosidases, using an expanded library of glycan substrates. We also investigate conditions in which several enzymes can be combined and we have applied this to prepare neo-glycoproteins exhibiting homogeneous glycosylation, whose structures were confirmed by liquid chromatography and mass spectrometry.

(91) Recombinant glycosidases as tools in glycan analysis
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Glycosidases are important tools in the analysis of the structural diversity of glycans. Many available glycosidases either have a broad or only partially defined substrate spectrum or are of
from microarrays and cleave neoglycopeptides containing a 2-aminobenzoic linker. [Supported by NIH Grant P41GM10369 to RDC.]

(93) An on-line multidimensional liquid chromatography strategy for coupling serum glycomic and glycoproteomic analytical data

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Changes in the N-glycosylation of serum proteins are known to be associated with cancer, in particular there is an increase in branching and sialylation and incorporation of sialyl LewisX epitopes (Colomb et al. 2012; Saldova et al. 2007). Many diseases are multi-systemic and observed changes in glycan and protein expression with disease progression are often interpreted independently of each other and out of context with biological networks. Thus there is a need for a systems biology approach in the development of technology for carbohydrate analysis. To address this we have developed a strategy for the analysis of serum glycoproteomes that incorporates the glycan moiety and the attached protein in the analysis. Here we describe the development of an automated multi-dimensional HPLC platform that enables high throughput affinity enrichment of target glycoproteins from plasma samples. Haptoglobin was chosen as a proof of concept investigation since previous studies have shown that the levels of this acute phase glycoprotein increase in various pathobiological processes including inflammation and cancer when compared with normal healthy individuals (Pompach et al. 2013). Depletion of albumin and IgG was coupled on-line with the enrichment of haptoglobin and tryptic digestion. SDS-PAGE analysis confirmed the successful capture of haptoglobin. This online targeted enrichment strategy coupled with online tryptic digest of the glycopeptide enables a dual function of characterizing the protein itself and the attached glycans. Furthermore, this platform can be adapted to enrich for any target glycoprotein and in this way the role of these glycoproteins in cancer and other diseases can be elucidated.

References


(94) Enzymatic N-Glycan Remodeling using α-2,6 Sialyltransferase

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ProZyme

Sialylation plays important roles in the development of biotherapeutic products; in particular it can have a large impact on product stability, efficacy and pharmacokinetics. A sialyltransferase enzyme (α-2,6 Sialyltransferase; ST6Gal-1) was used to increase sialylation, in vitro, of GalBl-4GlcNAc units on rituximab (mAb) and etanercept (Fc-Fusion Protein). The activity of ST6Gal-1 was examined via a timecourse of both in-solution and on-cartridge reactions using AssayMAP PA50 Cartridges (protein A). Glycan analysis was performed on all samples to determine the level of hypersialylation achieved at each timepoint. The release of N-Glycans, for all samples, was performed on AssayMap PA50 Cartridges by treatment with N-Glycanase®. Subsequent derivatization was performed using GlykoPrep® Rapid-Reductive-Amination™ 2-AB labeling. Chromatographic analysis was performed using a Waters ACQUITY UPLC®-FLR system. Additionally, linkage specificity was confirmed by treatment of the hypersialylated glycans with Sialidase A and Sialidase S.

(95) A Fully Integrated Method for LC-MS Analysis of Labeled and Native N-Glycans Released from Proteins and Antibodies

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Glycans present on therapeutic glycoproteins, glycolipids, and proteoglycans play significant roles in many biological processes. However, the complex nature of glycans poses challenges to their characterization. Liquid chromatography (LC) coupled to mass spectrometry (MS) has emerged as one of the most powerful tools for the structural characterization of glycans. Here we present a mixed mode stationary phase that provides superior selectivity and resolution compared to traditional modes. We have characterized the structures of native and labeled N-linked glycans from various proteins using this new column, hybrid quadrupole-orbitrap mass spectrometer and bioinformatics tools, thereby, enabling true high throughput workflow. The novel mixed mode columns separate glycans with unique selectivity based on charge, size, polarity and isomers. Structural analysis of both native and labeled glycans from proteins and antibodies were carried out successfully using this column by LC-MS method. Analysis of native glycans not only eliminates an extra step of reaction and cumbersome cleanup methods during labeling, but also retains the original glycan profiles without adding further ambiguity imposed by labeling reactions. Hybrid quadrupole-orbitrap mass spectrometer has been used for free glycan analysis. The primary advantage of this instrument is the ability to generate HCD fragmentation and detect them within the Orbitrap, providing HR/AM fragment ions. This allows for differentiation of near mass fragment ions which we observed to be useful for correctly assigning branching and linkage. In order to maximize both glycosidic and cross ring fragments, we incorporated step-collision energy. The use of LC-MS/MS for glycan analysis increases the complexity of data analysis due to the large number of MS/MS spectra. We incorporated bioinformatics tool to simplify data analysis. We extended this workflow to characterize glycans from various biological sources.

(96) Solid Phase Extraction of N-linked Glycopeptides Using Hydrazide Tip

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Glycoproteome contains valuable information where biomarkers may be discovered for disease diagnosis and monitoring. Nowadays, with the ever increasing performances of mass spectrometers, the emphasis is shifting to the sample preparation for better throughput and reproducibility. Therefore, to facilitate high throughput N-linked glycopeptide isolation, in this study, a novel hydrazide tip was devised and an integrated workflow of N-linked glycopeptide isolation using hydrazide tips was presented. Using bovine fetuin as a standard glycoprotein, the incubation time was determined for each major step of glycopeptide isolation. Using commercially available human serum, multiple parallel isolations of glycopeptides were performed using hydrazide tips with a liquid handling robotic system. We demonstrated that, with the hydrazide tips, the processing time was significantly decreased from 3-4 days to less than 8 h with excellent reproducibility. The hydrazide pipette tips have great potential in achieving automation of N-linked glycopeptide isolation for high throughput sample preparation when used in combination with liquid handling robotic systems.

(97) Microarray-based Epitope Analysis

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Human Mucin 4 (MUC4) is a high molecular weight O-glycoprotein expressed in epithelial tissues and abnormal expression has been seen in various carcinomas of the colon, pancreas, breast, and ovaries. Anti-MUC4 monoclonal antibody (mAb) is a promising tool for the study of MUC4 function and tumor diagnosis. 8G7 mAb is one of anit MUC4 mAbs raised against a KLH-conjugated MUC4 tandem repaet peptide STGDTPPLPTDTSSV. The 8G7 mAb has been used as a molecular tracer of MUC4 thanks to its strong reactivity against the MUC4 peptide and with native MUC4 from human tissues or cancer cells. However, fine epitope profiling on peptide segment and O-glicosylation pattern has not been reported so far. For unveiling them, we demonstrated epitope screening of 8G7 mAb by means of a microarray approach that we have developed recently. The most common MUC4 repeat sequence STGHATPLPTDTSSA was essential adoped for the peptide scaffold. A set of synthetic ladder peptides allows for the highlight of a crucial region on the repeat sequence. In addition, glycopeptide library with varied O-glicosylation patterns in terms of number, site, and structure will enable us to illuminate the ifurolence of
O-glycosylation(s) on the antibody reactivity. All peptides and glycopeptides were prepared by a standard solid-phase protocol, and then subjected to arraying on slides. Each synthetic probe carried 5-oxohexanoyl group at the N-terminus. An oxime bond is formed between a keto functionality of the probe and an aminooxy group on the surface of slides in chemoselective manner. The results of (G7 mAB binding assays with peptide and glycopeptide microarrays will be discussed.

(98) Effect of EUL-related nucleocytoplasmic lectin from Arabidopsis thaliana, ArathEULS3, on the root growth under salt stress
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Introduction: In the last decade several nucleocytoplasmic plant lectins have been discovered but very little is known with respect to the physiological role of these proteins. ArathEULS3 is a nucleocytoplasmic Euonymus related lectin that is expressed at low levels in Arabidopsis thaliana leaves. The recombinant ArathEULS3 exhibits specificity towards glycan structures having Galb1-4GlcNAc unit(s), such as Lewis X, Lewis Y or lactosamine, but the endogenous ligands for this lectin have not yet been identified (Van Hove et al. 2011; Fouquaert and Van Damme 2012). Aiming at a study of the physiological importance of the lectin we have generated transgenic plants after treatment with 130 mM NaCl for 8 days. However, salt stress significantly decreased the growth of roots in wild type, but overexpression of this lectin does not advance the development of Arabidopsis plants. Furthermore, the expression of ArathEULS3 was knocked down or increased in overexpression Arabidopsis plants.

References

(99) On the form and function of the Heparan sulfate code in Caenorhabditis elegans
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Heparan sulfate proteoglycans (HSPGs) are molecularly diverse extracellular molecules that control cell adhesion, motility and cellular responses by modulating protein signaling. Imaging studies as well as genetic and biochemical data indicate a heparan sulfate code that by way of its molecular complexity regulates cell behavior. However, direct correlation of glycan structure with function has been missing. Using the migration of the mecano-sensory neurons ALML, ALMR and AVM in Caenorhabditis elegans as a paradigm, we report here that the HSPGs Glypican-1/gpm-1, Glypican/lon-2, and syndecan/sdn-1, are required independently and non-redundantly for correct neuronal migration. Structural and genetic analyses show that each HSPG carries a distinct Heparan sulfate (HS) modification pattern. The most complex HS pattern is expressed in the nervous system and the least complex in the hypodermis and the intestine. We show that in the case of sdn-1/syndecan, the HSPG can act non-cell autonomously and that a single HS side chain on syndecan is necessary and sufficient for proper mecano-sensory cell position. We propose that the HS code that governs migration of mechanosensory neurons in Caenorhabditis elegans comprises at least two structurally distinct types of modification patterns that must act in concert for correct development.

(100) Keratan sulfate mediates zebrafish otolith
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Keratan sulfate (KS) is consistently found in the extracellular matrices that directly overlie sensory hair cells, yet little is known about the role these sugars have in mechanosensory function. We sought to define the expression of KS during early zebrafish otolith development, identify which enzymes were responsible for KS synthesis, and characterize otolith development in KS-deficient zebrafish embryos. We found that KS is expressed at the earliest stages of zebrafish otolith development. While KS is actively synthesized by the cells surrounding the otocyst beginning at 16 hours post fertilization (hpf), KS is not found in the otolith until 24 hpf, approximately 6 hours after the earliest otolith-localized protein, Oe90. KS forms a layered biomatrix that complements the expression of the otolith matrix protein OMP1. We identified b3gnt7b, chst2b, b4galnt2 and chst1 as putative KS-synthesizing enzymes there were expressed in the otic placode concomitant with early KS expression. Morpholino knockdown of b3gnt7b, chst2b, or b4galnt2 resulted in embryos with deformed or absent otoliths. We conclude that during early zebrafish otolith development, KS synthesis and secretion are temporally distinct. We identified genes responsible for all putative steps of KS synthesis during zebrafish otolith development; and using morpholino knockdown of each gene, we have demonstrated that KS is required for zebrafish otolith formation.
O-GlcNAc cycling is emerging as a key player linking nutrient flux to the epigenetic machinery. In Drosophila, OGT, the enzyme that adds O-GlcNAc on targets, is encoded by the polycomb group member sxc and is essential for appropriate hox gene repression. Although the null mutation is lethal, trans heterozygotes of sxc mutants survive and display homeotic transformations. In order to understand the role of excess O-GlcNAc on epigenetic regulation, we made a deletion in Oga gene coding for the removing enzyme OGA using P element excision. The Oga deletion (OGAdel.1) mutant displayed increased O-GlcNAc levels in comparison to wild type. Characterization of the deletion mutants revealed a gender-specific defect in which OGAdel.1 females exhibit low fecundity. Indeed, although the flies have increased triglyceride and glycogen levels, the females produce very few mature eggs, suggesting a starvation phenotype. Individual OGAdel.1 ovarioles displayed an increase in apoptosis in the germarium region, which could be a contributing factor in the low OGAdel.1 fecundity. We are in the process of determining whether the defects manifest themselves in the meiotic or mitotic region of the germarium. As O-GlcNAc cycling is known to be an important component of epigenetic control of gene expression, we analyzed the levels of some of the control genes that alter neural glycan expression. The Oga deletion (OGAdel.1) mutant displayed increased O-GlcNAc levels in comparison to wild type. Characterization of the deletion mutants revealed a gender-specific defect in which OGAdel.1 females exhibit low fecundity. Indeed, although the flies have increased triglyceride and glycogen levels, the females produce very few mature eggs, suggesting a starvation phenotype. Individual OGAdel.1 ovarioles displayed an increase in apoptosis in the germarium region, which could be a contributing factor in the low OGAdel.1 fecundity. We are in the process of determining whether the defects manifest themselves in the meiotic or mitotic region of the germarium. As O-GlcNAc cycling is known to be an important component of epigenetic control of gene expression, we analyzed the levels of some of the critical epigenetic modifications. We found a decrease in Histone 3 K4 trimethylation, while RNA polymerase II, phospho-serine 5 levels are increased in the OGAdel.1 mutants. The ovariole provides a tractable model to visually follow stem cell development. Therefore understanding the details of OGAdel.1 oogenesis may shed light on the role of O-GlcNAc cycling in stem cell differentiation and how dietary flux influences oogenesis.

A mutation in an E3 ubiquitin ligase alters neural-specific glycosylation in the Drosophila embryo

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Glycoprotein glycosylation is essential for normal development and viability in all multicellular organisms. However, the pathways and mechanisms that control tissuespecific glycan expression are not well understood. Drosophila melanogaster embryos express a family of neural-specific, core α3-fucosylated N-linked oligosaccharides called HRP epitopes. A previously described mutation in the tollo/toll-8 gene (a member of the Toll-like receptor family) abolishes neural expression of HRP-epitopes and is part of a transcellular signaling pathway that induces neural-specific glycosylation in the embryonic nervous system. In order to further characterize this pathway, a random mutagenesis screen was performed to find additional genes that alter neural glycan expression. The first mutation characterized from this screen identified a Ser/Thr kinase called Sff (sugar-free frosting) that interacts genetically with tollo and modulates Golgi trafficking in neurons. Another mutation recovered from this screen, designated MS16, exhibits significant reduction of HRP-epitope expression and shares other phenotypic characteristics associated with both tollo/toll-8 and sff; MS16 also interacts genetically with both tollo/toll-8 and sff. Glycomic analysis verified the reduction of HRP-epitope glycans in MS16 embryos and also demonstrated that the mutation shifts the total N-linked glycan profile toward increased complexity. Genetic mapping and molecular characterization placed the MS16 mutation in the roc2 gene, an E3 ubiquitin ligase previously shown to be essential for neural development. At the protein level, not all glycoproteins that normally carry the HRP-epitope are equally affected by the MS16 mutation, indicating that Roc2 functions to ensure the proper glycosylation of a subset of proteins.

Expression and roles of galectins (DrGRIFIN and DrGal1) in zebrafish eye development

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Galectins, a family of β-galactoside-binding proteins, participate in multiple biological processes, such as early development, tissue organization, immune regulation, and tumor evasion and metastasis. The detailed mechanisms of their biological roles, however, still remain unclear. Ongoing studies in our laboratory are aimed at elucidating their roles in eye development using the zebrafish as a model system. Through in silico analysis we identified in zebrafish a prototype galectin sequence that in BLAST analysis produced the highest match with the mammalian GRIFIN (Galectin related inter-fiber protein). Like the mammalian equivalent, DrGRIFIN is expressed in the lens fiber cells, as revealed by whole mount in situ hybridization and immunostaining of 2 dpf (days post fertilization) embryos. As evidenced by RT-PCR, it is weakly expressed in the embryos as early as 21 hpf (hour post fertilization) but strongly at all later stages tested (30 hpf and 3, 4, 6, and 7 dpf). In adult zebrafish tissues, however, DrGRIFIN is also expressed in oocyte, brain, and intestine. Unlike the mammalian homologue, DrGRIFIN contains all amino acids critical for binding to carbohydrate ligands and its activity was confirmed as the recombinant DrGRIFIN could be purified to homogeneity by affinity chromatography on a lactosyl-Sepharose column. In addition, in a previous study on retinal injury in zebrafish, we identified the prototype galectin DrGal1-L2 as a key factor in retinal regeneration. In preliminary studies we investigated the effect of knocking down the expression of either DrGRIFIN or DrGal1-L2, using morpholino (MO)-derived antisense oligonucleotides to block the protein translation. The zebrafish embryos before the 4-cells stage were microinjected with gene-targeting MO or its mismatch (MM) oligonucleotides as control. The embryos were collected at 24, 48, or 72 hpf for phenotypic observation, fixed for histological staining, processed for protein extractions for Western
Interaction of ganglioside GD3 with EGF receptor maintains the self-renewal ability of murine neural stem cells

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Mounting evidence supports the notion that gangliosides serve regulatory roles in neurogenesis; little is known, however, how they function in neural stem cells (NSCs) fate determination. We previously demonstrated that GD3 is a dominant ganglioside in mouse NSCs, and may serve as a surface marker of NSCs. Since EGF is an important mitogen for NSCs, we hypothesized that GD3 may act with EGF receptor (EGFR) in mediating EGF function. Thus, we investigated the role of GD3 in NSCs of GD3-synthase knockout (GD3S-KO) mice and their wild-type littermates. NSCs, prepared from the brain of embryonic mice and the subventricular zone (SVZ) of adult mice using the neurosphere method. The NSCs of GD3S-KO mice showed reduced ability to form neurospheres, compared with those from the WT animals. The decreased self-renewal ability of the GD3-NSCs was accompanied by a reduced EGF receptor expression and an increased degradative rate of EGFR signaling, which could be rescued by over-expression of GD3S gene in the GD3S-KO mice. We also found that EGFR was present in the lipid raft fractions of NSCs of WT, but not in the knockout animals; EGFR and GD3 were colocalized in the wild-type NSC plasma membranes; polystyrene beads coated with GD3 bound to more EGFR from wild-type than from GD3S-NSC; EGFR could be detected in NSC lysate immunoprecipitated with an anti-GD3 antibody (R24) from the wild type but not from the GD3S-KO mice; treatment of NSCs with EGF resulted in more EGFR in GD3S-NSCs to translocate through the endosomeal-lysosomal degradative pathway, rather than the recycling pathway. These data were corroborated by in vivo studies which revealed impaired neurogenesis in the SVZ of GD3S-KO mice by a reduced number of cells with BrdU incorporation in the SVZ of GD3S-KO mice, and decreased co-staining of the BrdU-positive cells with the NSC marker Sox2 and GFAP in GD3S-KO brain sections. We conclude that GD3 interacts with EGFR in the lipid raft of plasma membranes of NSCs, and this interaction is responsible for sustaining the EGFR and the downstream signaling and in maintaining the self-renewal ability of NSCs.

A model for N-glycolyneuraminic acid expression and function in mammalian muscle

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Approximately 2-3 million years ago, our human ancestors lost the function of CMAH -- a gene that remains fully intact in our most closely related species, the bonobos and chimpanzees (Chou et al. 1998). CMAH codes for a mono-oxygenase that hydroxylates the CMP donor form of the most common sialic acid in mammals called N-acetylenuraminic acid (Neu5Ac), and converts it into N-glycolyneuraminic acid (Neu5Gc). Expression of Neu5Gc varies greatly by tissue type and across different species. For example, we have found that while the sialic acid content of mouse muscle is ~55% Neu5Gc, western dog muscle is comprised of 1-2% Neu5Gc, which is more similar to humans. This difference in sialic acid content may be an explanation for the difference in muscular dystrophy severity observed between mice, dogs, and humans. In fact, we have previously shown that the loss of Cmah in mice increases the disease severity of the models for Duchenne Muscular Dystrophy (Chandrasekharan et al. 2010) and Limb Girdle Muscular Dystrophy type 2D (Martin et al. 2013). Thus this difference in muscle Neu5Gc content has an as yet unknown effect on muscle function and disease. Currently, we are exploring the role of Neu5Gc in a well-characterized mouse myoblast cell line (C2C12), which was found to upregulate Cmah during differentiation into myotubes. Although C2C12 myoblast cells contain only ~3% Neu5Gc, cells differentiated in human serum contain up to 40% Neu5Gc on their cell surface. This is the first stable cell line discovered to drastically increase cell surface expression of Neu5Gc during differentiation without requiring exogenous metabolic incorporation. This will provide us with a model for studying the effect of cell surface sialic acid differences in culture without any of the unknown metabolic effects of sialic acid feeding. To further explore the role of Neu5Gc expression in muscle differentiation, we are also utilizing the CRISPR/Cas9 custom guided RNA system (Mali et al. 2013) to engineer a C2C12 line that will lack a functional Cmah transcript.

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Specific Interaction between Wzz and O-polysaccharide Provides Novel Insights into Regulation of Lipopolysaccharide Modality

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The chain length regulation of O-polysaccharide (O-PS) region in lipopolysaccharide (LPS) is often associated with virulence in Gram-negative bacteria. However, the mechanistic details of O-PS chain length regulation are largely unknown. Herein, we provide conclusive evidences on the existence of a direct interaction between O-PS and its chain length regulator Wzz on the basis of our pull-down and surface plasmon resonance (SPR) analyses. We also observed the interaction between Wzz and its natural substrate undecaprenol-PP-linked O-PS, and further demonstrated that structural changes in O-repeat unit of O-PS affect LPS chain distribution pattern. In addition, Wzz homologues exhibit different binding specificity towards O-PS in a chain length-dependent manner. To understand the details in the binding, we employed molecular docking online sever to simulate this interaction, which reveals that O-PS binding sites are contributed by two adjacent subunits and predominantly located inside the bell-shaped Wzz oligomer. Taken together, a new model is proposed in which the specific interaction of Wzz and O-PS plays a key role in O-PS chain length determination.

This research is supported by NIH R01 GM085267 for financial support.

(107) **Biosynthesis of the Common Polysaccharide Antigen of Pseudomonas aeruginosa:** Characterization and role of GDP-D-rhamnose: GlecNAc/GalNAc-diphosphate-lipid D-rhamnosyltransferase WbpZ

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The Common Polysaccharide Antigen (CPA, A-band O antigen) in *Pseudomonas aeruginosa* PA01 contains D-rhamnose residues in α-2 and α-3 linkages. Three putative glycosyltransferase genes are present in the O antigen gene cluster associated with CPA synthesis. In this study, we have carried out biochemical characterization of the D-rhamnosyltransferase encoded by the *wbpZ* gene that was proposed to catalyze the second step in O antigen repeating unit synthesis. Our data showed that WbpZ transferred a Rha residue from GDP-D-rhamnose to GlecNAc-diphosphate-lipid acceptor substrates but was less specific than other O antigen glycosyltransferases and also accepted GalNAc-diphosphate lipids. Anthracenyl-undecyl-linked GalNAc and GlecNAc derivatives formed good acceptor substrates which could be employed in non-radioactive, fluorescent assays. WbpZ was most active with the donor substrate GDP-D-Rha and had an unusually high pH optimum. The enzyme did not require divalent metal ions for activity, consistent with WbpZ being a retaining glycosyltransferase of the GT4 family with a GT-B fold. The biochemical characterization of this D-rhamnosyltransferase is a critical step in the development of CPA synthesis inhibitors to combat *Pseudomonas aeruginosa* infections. This work was supported by a Discovery grant of the Natural Science and Engineering Research Council of Canada.

(108) **Biochemical evidence for an alternate pathway in N-linked glycoprotein biosynthesis**

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Asparagine-linked glycosylation is a complex protein modification conserved among all three domains of life. Much is known about N-glycan assembly in eukaryotes and selected bacteria, where a polyprenyl-diphosphate-dependent pathway is initiated by a phospho-glycosyl transferase and elaborated with a series of glycosyltransferases to generate a polypeptide-PP-linked glycan. Specifically, in the *Campylobacter jejuni* bacterial pathway, the oligosaccharyl transferase (OTase), known as PglB, utilizes the α-linked undecaprenol-PP-heptasaccharide substrate as the glycosyl donor and transfers the glycan to the asparagine residue of acceptor proteins. Unlike in eukaryotes and bacteria, the biochemical details of archaeal N-linked glycosylation have remained elusive. We report here the polyprenyl-monophosphate-dependent pathway used by selected archaea through the in vitro analysis of N-linked glycosylation from the marine methanogenic archaeon *Methanococcus voltae*. The archaeal OTase, known as AglB, utilizes the α-linked dolichyl-P-trisaccharide substrate as the glycosyl donor for transfer to the acceptor protein. This dolichyl-P-glycan is generated by an initial retaining glycosyltransferase (AglK) and elaborated by additional glycosyltransferases (AglIC and AglA) to afford Dol-P-GlcNAc-Glc-2,3-diNAcA-ManNAc(6Thr)A. Despite the sequence homology to C. jejuni PglB and other bacterial or eukaryotic OTases that exploit polyprenyl-PP-linked substrates, the *M. voltae* AglB efficiently transfers disaccharide to model peptides from the Dol-P-GlcNAc-Glc-2,3-diNAcA monophosphate. While this archaeal pathway affords the same asparagine-linked β-glycosyl amide products generated in bacteria and eukaryotes, these studies provide the first biochemical evidence revealing that despite the apparent similarities of the overall pathways, there are actually two general strategies to achieve N-linked glycoproteins across the domains of life. There is a strong need for detailed studies on the mechanistic and functional significance of archaeal adaptations of N-linked glycosylation, especially to probe the structural, conformational, or chemical differences between AglB, PglB, and the other OTases that allow AglB to utilize these unique polyprenyl-P-linked substrates. Biophysical studies exploring peptide and Dol-P-glycan binding to AglB should prove particularly illuminating.

(109) **MicroRNA Define the Glycocode: Identification of Critical Glycogenes That Shape The Cell**

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Cell surface carbohydrates create an intricate source of biological information which is dynamically altered by cells during developmental processes and used by pathogens during host invasion. Mapping of the glycocode and identification of critical glycan altering enzymes in biological processes is confounded by the complexity and redundancy of the glycan biosynthetic machinery. Recently we showed that...
microRNA act as a major regulator of the human glycome by targeting critical nodes within the glycan biosynthesis pathway. In line with our findings, we utilize miRNA target prediction software and our glycobiology data to identify highly regulated glycosyltransferases. Among the predictions, we identified a strong association of an unusual glucosyltransferase B3GALTL with miRNA regulators of epithelial to mesenchymal transition (EMT), miRNA-200 family. We confirmed regulation of B3GALTL by the miRNA-200 family, and we show that B3GALTL knockout induced a change in morphology from mesenchymal to epithelial in mesenchymal breast cell line MB-231. Taken together, our data validates miRNA-based mapping of the glycome and micromics data to identify highly regulated glycosyltransferases. Among the predictions, we identified a strong association of an unusual glucosyltransferase B3GALTL with miRNA regulators of epithelial to mesenchymal transition (EMT), miRNA-200 family. We confirmed regulation of B3GALTL by the miRNA-200 family and we show that B3GALTL knockout induced a change in morphology from mesenchymal to epithelial in mesenchymal breast cell line MB-231. Taken together, our data validates miRNA-based mapping of the glycome and identifies B3GALTL as an important regulator of EMT.

(110) New epigenetic factors for brain-specific glycosyltransferase gene expression
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Glycosyltransferase gene is regulated in a tissue-specific manner which governs tissue-specific glycosylation, but little is known about the regulation mechanism of glycosyltransferase genes. To tackle these questions, we focused on several epigenetic factors, especially histone-modifying enzymes which are involved in histone acetylation and O-GlcNAcylation. First, using a chemical inhibitor, overexpression and knockdown experiments, we found that HDAC11 is specifically involved in epigenetic silencing of Gnt-IX but not involved in other glycan genes. It suggests that HDAC11 is somehow responsible for epigenetic repression through histone de-acetylation in a glyco-specific way. Next, we focused on O-GlcNAc transferase (OGT)-TET complex, because it was recently reported that this complex is required for activation of target genes through O-GlcNAcylation of histone. Actually, we found that both OGT and TET3 molecules are required for Gnt-IX transcription and that OGT binding to Gnt-IX promoter is disrupted by TET3 depletion. It suggests that TET3-OGT complex is involved in Gnt-IX activation through O-GlcNAcylation of histones. Collectively, it suggests that Gnt-IX, unlike other glycogenes, is highly susceptible to epigenetic regulation, and we newly identified epigenetic factors (HDAC11 for silencing and TET3-OGT for activation) which are selectively required for Gnt-IX expression. This work would give us a new clue to understand as yet unknown mechanisms for glycosyltransferase gene expression.

(111) Protein O-glycosylation in secretory apparatus structure and endoplasmic reticulum stress
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Protein N-linked glycosylation is responsible for mediating proper protein folding and quality control within the secretory apparatus. Aberrations in this process lead to endoplasmic reticulum (ER) stress and trigger the unfolded protein response (UPR). Previously, we found that an enzyme that initiates mucin-type O-linked glycosylation (PGANT35A) is required for proper formation of the Drosophila respiratory system. In the present study, we demonstrate by electron microscopy that mutations in this glycosyltransferase result in alterations of the secreted apical and luminal structures in the developing tracheal system. We also found tracheal cells of pgant35A mutants showed an increase in the amount of ER present relative to wild type, suggesting that loss of this transferase may result in an ER stress response. In support of this, RNAi to pgant35A in Drosophila cell culture resulted in an expansion of the ER as well as the induction of genes involved in ER stress. Additionally, we found that pgant35A gene expression was up-regulated in response to other inducers of ER stress, suggesting a role for O-glycosylation in the ER stress response. Further analyses revealed functional ER stress responsive elements in the 5′ regulatory region of the pgant35A gene. Taken together, our results provide evidence that O-glycosylation is a component of the ER stress response and provide insight into the role of mucin-type O-glycosylation in the regulation of secretory events occurring during development.

(112) Investigating the function of Htm1p in the N-glycoprotein quality control pathway
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During eukaryotic translation, nascent membrane and secretory proteins are translocated into the endoplasmic reticulum (ER) lumen in an unfolded state and are folded with the help from ER chaperones. While correctly folded proteins are sorted for further modifications and export, terminally misfolded proteins are recognized and retro-translocated to the cytoplasm for proteasomal degradation in a process known as ER-associated protein degradation (ERAD). In Saccharomyces cerevisiae, Htm1p is proposed to recognize and mark the misfolded ER luminal N-glycoproteins by specifically removing a terminal mannose from the glycans on the misfolded N-glycoproteins. The resulting glycan is recognized by the downstream lectin complex to permit retrotranslocation and degradation of the misfolded proteins. However, the question of how Htm1p achieves its specificity to misfolded species remains unanswered. In this study, we combined genetic and biochemical approaches to investigate the molecular mechanisms of how Htm1p recognizes the substrates and processes the glycans, and what the critical properties of the misfolded substrates are.

(113) The Structural Basis of the Recognition of Di-glucosylated N-glycans by the ER Lectin Malectin
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Malectin is a unique protein first detected in Xenopus laevis but soon found to be highly conserved in animals. Structure
determination of the xenopus protein by NMR revealed a close structural similarity to carbohydrate-binding modules (CBMs) of bacterial hydrolases that recognize glucan polysaccharides. Glucose binding was corroborated by exploratory isothermal calorimetry (ITC) using maltose (Glcα1,4Glc; K \approx 50 \mu M), hence the name malectin. The results of follow-on ITC showed a preference for nigerose (Glcα1,3Glc; K \approx 26 \mu M). The demonstration that malectin is located in the endoplasmic reticulum (ER) led to carbohydrate microarray analyses using a neoglycolipid (NGL)-based microarray that included glucan oligosaccharide fragments and a diverse range of oligosaccharide probes of mammalian-type sequences, among them the mono-, di- and tri-glucosyl high-mannose N-glycans that occur in the ER. The microarray analyses corroborated the glucan-binding property of malectin, and revealed a remarkable selectivity for the 1,2 1 15 di-glucosyl high-mannose N-glycan sequence, Glc Man (D1)GlcNAc. Recent H N-HSQC experiments and ITC using the tetrascarhide Glc Man (K 2 7 1 2 2 3 4 \sim 12 \mu M) and with Glc Man GlcNAc (K \sim 7 \mu M) have corroborated recognition of the terminal di-glucose sequence Glcα1,3Glc and indicated that most d 2 9 2 d 3 of the affinity of malectin is toward the di-glucosyl cap.

Here we will report high-resolution crystal structures of human malectin in complex with disaccharide nigerose and pentasaccharide Glc Man, 2 3 representing the D1 arm of the di-glucosyl high-mannose core-mannosyl N-glycan fragment. The fine specificity of human malectin towards di-glucosylated sequences is also evaluated using NGL-based microarrays of various analogs of the di-glucosyl N-glycan Glc2 Man9 GlcNAc2 with different degrees of truncation, chemically or chemo-enzymatically generated, namely, Glc2 Man8 GlcNAc2 (B and C isoforms), Glc2 Man7GlcNAc1, Glc2 Man4 GlcNAc2, Glc Man2, Glc3 Man2, and Glc2 Man1. These studies elucidate at molecular level the size of the determinant recognized by malectin, and the participation of the adjacent mannosyl residues on the D1 arm of the di-glucosyl high-mannose N-glycan ligand. Supported by Wellcome Trust and Fundação para a Ciência e Tecnologia.

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(114) Crystal Structure of Glucosidase II’s Mannose 6-Phosphate Receptor Homology (MRH) Lectin Domain
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In the endoplasmic reticulum (ER) of eukaryotic cells, N-glycan structures attached to proteins are modified as part of a quality control mechanism ensuring the correct folding of these secretory pathway proteins. In most eukaryotes, Glc3Man9GlcNAc2 is transferred from a dolichol pyrophosphate derivative to nascent polypeptides in the ER. Glucosidase I immediately removes the terminal glucose of the glycan revealing two inner glucose moieties that are in turn trimmed by the catalytic α-subunit of the heterodimeric glucosidase II (GII). GII’s hydrolytic activity is regulated by the mannose binding activity of the mannose 6-phosphate receptor (MPR) homology domain (MRH domain) contained within GII’s β-subunit. Removal of the middle glucose creates a monoglucosylated glycan that is recognized by lectin-chaperones, calnexin (CNX) and calreticulin (CRT), known to enhance polypeptide folding and ER retention of misfolded glycoproteins. Additional opportunities for folding are available through the reglucosylating activity of UDP-Glc:glycoprotein glucosyltransferase (UGGT) which recognizes non-native conformations and regenerates monoglucosylated glycoproteins capable of reassociating with CNX/CRT. GII also removes glucose added by UGGT irrespective of the protein’s folding status. The opposing activities of GII and UGGT allow for cycles of deglucosylation-reglucosylation to occur until either the glycoprotein acquires its native conformation or it is marked for degradation in a process known as ER-associated degradation (ERAD). MRH domains have long been investigated in the context of trafficking acid hydrolases to endosomal/lysosomal compartments and more recently have been studied in the resident ER proteins OS-9 and XTP3-B, which function in ERAD, and in the non-catalytic subunit of Golgi GlcNAc-phosphotransferase, which modifies acid hydrolases for their subsequent interaction with MRPs. Optimal GII deglucosylation activity requires a functional GIIB MRH domain and nascent glycoproteins bearing Man9-containing glycan chains. To better understand the role of GIIB MRH domain, we previously determined the solution structure of Schizosaccharomyces pombe GIIB’s MRH domain that revealed the conserved MRH fold observed in the MPRs and OS-9.

We now report a 1.6Å crystal structure of S. pombe GIIB’s MRH domain in the presence of bound mannose and compare its shallow binding pocket with the phosphorylated mannose-specific and Manα1,6Manα1,6Man-specific MRH domain structures of MPRs and OS-9, respectively.

(115) The Endoplasmic Reticulum Located ppGalNAc-T18 (GALNTL4) is Involved in Cell Survival and Apoptosis
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Mucin-type O-glycosylation of proteins is one of the most important post-translational modification that plays essential roles during eukaryotic physiological and pathological processes. Recently, we found that ppGalNAc-T18, which is a member of vertebrate-specific Y subfamily of UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferases (ppGalNAc-Ts) and also known as polypeptide GalNAc transferase-like protein 4 (GALNTL4), shows chaperone-like function in regulating protein O-glycosylation and plays an essential role in maintaining endoplasmic reticulum (ER) homeostasis and cell survival.
ppGalNAc-T18 localizes in the ER and lacks classical GalNAc-transferase activity, it can selectively modulate the in vitro GalNAc-transferase activity of ppGalNAc-T2 and -T10, and it can interact with ppGalNAc-T2 or mucin substrate. In this study, we found that knockdown of ppGalNAc-T18 affected cellular survival by activating the ER stress response, while overexpression of ppGalNAc-T18 protected cells against apoptosis induced by pathological stimuli. Moreover, we found that ppGalNAc-T18 was upregulated in the apoptotic neuronal cells in the brain of mice in vivo after intraperitoneal injection of lipopolysaccharide and in apoptotic neuron-like PC12 cells treated with inflammatory cytokines in vitro. These results indicated that ppGalNAc-T18 was an essential protein for maintaining ER functions and cells survival. Our work demonstrated that ppGalNAc-T18, an ER-localized ppGalNAc-T-like protein, not only could be involved in regulating the O-glycosylation, but also participated in maintaining ER homeostasis, it has important roles in cells survival and apoptosis.

(116) Role of Cosmc and O-Glycans in Endothelial Cells
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O-glycoproteins participate in essential cellular processes such as signaling and communication, cell adhesion, vascular development and lymphangiogenesis, immune surveillance, inflammation, and endocytosis. Cosmc is a molecular chaperone that is required for expression of active T-synthase, the only enzyme that galactosylates the Tn antigen (GalNAcα1-Ser/Thr-R) to form core 1 Galβ1-3GalNAcα1-Ser/Thr (T antigen) during mucin type O-glycan biosynthesis. Loss of Cosmc is associated with loss of T-synthese. Because mucin-type O-glycans (O-glycans) are highly expressed in vascular endothelial cells (EC), we studied the functions of core 1-based O-glycoproteome in murine lung endothelial cells. We isolated Tn(+) and Tn(-) murine lung endothelial cells from +/- endothelial/ hematopoietic cell (EHC)-specific Cosmc-knockout mosaic female mice (EHC-Cosmc). Tn(-) ECs express CD31 but not Tn antigen, while Tn(+) ECs have both CD31 and Tn expression. RT-PCR shows that whereas Tn(+) ECs have lost Cosmc expression, the cells express normal levels of VWF, P-selectin, and ADAMTS 13. The Tn(+) EC lost PNA lectin binding, which binds to core 1 O-glycans, but show high binding to HPA lectin, which binds to the Tn antigen. We performed mass spectrometry profiling of the O-glycans and N-glycans in Tn(-) and Tn(+) EC. The O-glycans from Tn(-) EC have compositions that indicate they are mainly mono-and di-sialylated core 1, as well as mono- and di-sialylated core 2 structures. Importantly, no significant levels of extended O-glycans were found in Tn(+) EC, demonstrating that O-glycan biosynthesis is disrupted after Cosmc deletion. The N-glycan profile from Tn(+) EC is similar to that from Tn(-) EC, demonstrating that N-glycans remain unchanged with Cosmc expression. The generation of Tn(+) EC will promote future studies on the role of O-glycosylated glycoproteins in these cells.

(117) A new Congenital Disorder of Glycosylation caused by a mutation in SSR4, the signal sequence receptor 4 protein of the TRAP complex
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Nearly 50 Congenital Disorders of Glycosylation (CDG) are known, but many patients biochemically diagnosed with CDG do not have mutations in genes known to cause CDG. To identify these mutated genes, we used the exome sequencing on DNA trios corresponding to the patient and parents DNA. Using this method, we identified a de novo variant in the X-linked SSR4 gene which encodes a protein of the heterotetrameric translocon-associated protein (TRAP) complex. Here, we describe a sixteen year old male with type I CDG who was born with microcephaly, and developed intellectual disability, gastroesophageal reflux and a seizure disorder. The c.316delT identified mutation causes a p.F106Sfs*53 in SSR4 and also reduces expression of other TRAP complex proteins. The glycosylation marker Glyc-ER-GFP was used to confirm the underglycosylation in fibroblasts from the patient. Over-expression of the wild-type SSR4 allele partially restores glycosylation of the marker and of the other members of the TRAP complex. This is the first evidence that the TRAP complex, which binds to the olosaccharyltransferase complex, is directly involved in N-glycosylation. This work was funded by the NIH R01DK55615 and The Rocket Fund.

(118) The reverse sialylation properties of the human α(2,3) sialyltransferase ST3Gal-I mediates sialoglycan biosynthesis
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The capping of mammalian cell-surface glycoconjugates by α(2,3)-linked sialic acid often regulates receptor-ligand recognition. Five α2,3 sialyltransferases, named ST3Gal-I, -II, -III, -IV and VI, catalyze the terminal modification of core 1,3GlcNAc (Type-I), 1,4GlcNAc (Type-II) or Galβ1,4GlcNAc (Type-III) terminal structures. Such studies showed that sialylated Type-I glycans (Neu5Acα2,3Galβ1,3GlcNAc) are synthesized by ST3Gal-I, -II, -III, -IV and -VI. ST3Gal IV and VI, and to a lesser extent ST3Gal-III, formed sialylated Type-II glycans (Neu5Acα2,3Galβ1,4GlcNAc). ST3Gal I, -II and also ST3Gal-IV synthesized sialylated Type-III glycans (Neu5Acα2,3Galβ1,3GlcNAc). In addition to the conventional 'forward' sialylation reaction above, ST3Gal-I and to a lesser
extent ST3Gal-II catalyzed the reverse synthesis of CMP-Neu5Ac from 5′-CMP in the presence of sialic acid donors containing the Neu5Acα2,3Galβ1,3GalNAc unit. This reaction is called ‘reverse’ sialylation. ST3Gal-I also readily catalyzed the exchange/ swapping of sialic acid residues between sialylated Type-III glycoconjugates and different forms of activated sialic acid, including the glycolyl form i.e. CMP-Neu5Gc. This reaction is called ‘exchange sialylation’. Finally, the analysis of soluble glycosyltransferases in human blood plasma reveals the presence of both ‘reverse’ and ‘exchange’ sialylation activity. Overall, in addition to the unidirectional transfer of sialic acid from CMP-Neu5Ac to various glycoprotein acceptors in the Golgi, the ‘reversible’ enzyme activity of ST3Gal-I may also regulate the pattern of α(2,3)-linked sialoglycans in blood.

(119) Detecting Alterations of Golgi Resident Glycosylation Enzymes Via Quantitative Proteomics: Analysis of Enriched Golgi Membranes
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Advances in glycomics, which aim to map alterations of cell surface glycosylation during pathophysiological development have led to identifications of glycan epitopes as diagnostic biomarkers as well as targets for therapeutic strategies. However, little is known about in vivo functions of these disease-associated glycan epitopes and how the synthesis of these glycan epitopes are regulated during disease development. Glycan chains are synthesized by the concerted actions of glycosylation enzymes aligned along the secretory pathway. A way to measure the alteration of the cell surface glycan profile can be inferred by the mRNA expression of glycosylation enzymes. Apart from gene expression, these glycosylation enzymes are also regulated at the protein level by post-translational modifications and complex formation, which cannot be readily implied from mRNA expression data. A full understanding of biosynthetic regulation of glycosylation of proteins thus requires study of the endogenous glycosylation enzymes at the protein expression level. Analysis of the glycosylation enzymes is hindered by the fact that most of them are Golgi resident membrane proteins and low in abundance. Advances in mass spectrometry based proteomics provide promising opportunities to tackle such low analytical limits if coupled with appropriate sample preparation. In this study, we prepared Golgi enriched membranes from two colonic cancer cell lines, SW480 and SW620, and demonstrated that this step is necessary to enhance the identification of Golgi resident glycosyltransferases. A total of 19 Golgi resident glycosyltransferases involved in protein N-and O-glycosylation were identified in data set originated from SW480 and SW620 Golgi membranes. Based on these results, we established selected reaction monitoring (SRM) methods that enabled sensitive and quantitative comparison of targeted glycosylation enzymes between cells. The alteration of the glycosyltransferases thus obtained were compared with mRNA profiling of their expression levels as well as with the structures of the glycans that are synthesized. The data reveal subtle regulation of glycosylation enzymes at the cellular level.

(120) Characterization of UDP-Gal: GalNAcα-PP-R β1,3-Gal-transferase WbwC, a T-synthase from STEC O104
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Enterohemorrhagic strains of E. coli display O-antigenic polysaccharides on their outer membranes that play important roles in interactions with the environment. The O104 antigen contains a Galβ1-3GalNAcα-disaccharide at the reducing end of the O antigen repeating unit. We cloned and identified the wbwC gene involved in the synthesis of this linkage. A synthetic substrate analog, GalNAcα-diphosphate-phenylundecyl, was used as an acceptor analog and UDP-Gal as a donor substrate to demonstrate that wbwC encodes a Gal-transferase. The WbwC enzyme product was isolated by HPLC and analyzed by MALDI-MS, NMR, galactosidase and O-glycanase digestion. The results showed that the expected Gal β 1-3-GalNAcα linkage was synthesized. The disaccharide is identical to mammalian O-glycan core 1 and was recognized by anti-T antibodies as a T antigen. This identifies WbwC as a bacterial T synthase: UDP-Gal: GalNAcα-diphosphate-lipid β1,3-Gal-transferase. Sequence analysis of WbwC and similar enzymes revealed a conserved DxDD motif. Mutagenesis confirmed the importance of Asp91 and to a lesser degree Asp94 in catalysis while mutation of Asp93 to Ala did not affect activity, confirming that in this enzyme a DxxD motif is functionally important instead of the DxD motif which is 2+ essential in many other inverting enzymes. Purified WbwC requires divalent cations (Mn²⁺) for activity, has a broad pH optimum and is specific for both UDP-Gal as the donor substrate and GalNAcα-diphosphate-lipid as the acceptor substrate. WbwC can be inhibited by bis-imidazolium salts having aliphatic chains of 18 to 22 carbons, which have potential as adjuvant antibiotics. This work helps to elucidate mechanisms of polysaccharide synthesis and provides a new technology for anti-cancer and anti-bacterial vaccine synthesis. In addition, WbwC is a potential target for anti-bacterial drug development. This work was funded by a Discovery grant from the Natural Science and Engineering Research Council of Canada, a grant from the Canadian Institutes of Health Research, by the National 973 Program of China, National Natural Sciences Foundation of China and the National Key Program for Infectious Diseases of China.

(121) A structural and biochemical model of processive chitin synthesis
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Chitin synthases (CHS) produce chitin, an essential fungal cell wall component, and are an attractive target for the development of
antifungal drugs. There is a need to understand the molecular mechanism of processive synthesis of chitin and to discover new inhibitors of this enzyme class. However, these large multi-transmembrane domain proteins have so far resisted expression and purification hampering compound screening and structural studies. We have identified the bacterial glycosyltransferase NodC as a useful model system to study the structure and reaction mechanism of chitin synthesis. We present a novel non-radioactive, high-throughput compatible assay to measure chito-oligosaccharide synthesis by NodC. We determined the overall membrane topology biochemically using a combined fusion protein approach and confirmed it by generating a structural model of NodC. A series of NodC residues -conserved amongst CHS- were targeted by mutagenesis and investigated for their effect on NodC activity. Known CHS inhibitors were tested for inhibitory properties against NodC. We show that the bacterial NodC proteins are useful models to study the structure and mechanism of CHS and for the identification of novel inhibitors.

(122) Structure-function study of MGD1, the major galactolipid synthase in Arabidopsis
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Galactolipids, such as monogalactosyldiacylglycerol (MGD) and digalactosyldiacylglycerol (DGDG), are a unique lipid class ubiquitously found in photosynthetic organisms, from cyanobacteria to land plants. MGDG and DGDG account for ~50% and 25% of the membrane lipids in plant chloroplasts, respectively. These galactolipids are essential to the biogenesis of plastids and photosynthetic machinery. In plants, MGDG synthesis is catalyzed in a single step by a MGDG synthase (called MGD), which transfers a galactosyl residue from UDP-galactose to diacylglycerol (DAG). MGD1 is the major galactolipid synthase in Arabidopsis. It is a monotopic protein localized in the plastid envelope. The catalytic domain of MGD1 [aa 137-533] has been successfully expressed as an active and soluble form into E. coli. The purification protocol we developed typically yields milligram quantities of pure and homogenous protein material suitable for crystallization and biochemical studies [Rocha et al., 2013]. We also revisited the conditions for activity tests and effects of known positive effectors of MGD1 such as phosphatidic acid and phosphatidylglycerol [Dubots et al., 2010; Rocha et al., 2013]. The crystal structure of the catalytic domain of MGD1 has been obtained free and in complex with UDP. MGD1 displays the canonical GT-B fold with two distinct Rossmann-type domains. However, two specific structural features, not seen in related structural homologues, are observed in MGD1: (i) the presence of a large disordered loop encompassing residues 182 to 230 in the N-terminal domain and (ii) an extra 2-stranded β-sheet in the C-terminal domain. The disordered loop is believed to be essential for acceptor recognition and/or membrane interaction. Exploration of the active site gives insight into residues critical for binding UDP-Gal and clues for DAG recognition.

References
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(123) The WD40-repeat domains of Dictyostelium Skp1 α-galactosyltransferase (AgтA) mediate Skp1-substrate activation of AgtA and modulate Skp1 activity in cells
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Skp1’s role as an adaptor protein that links Cullin-1 to F-box protein in E3 Skp1-Cullin-F box protein (SCF) ubiquitin ligases has been well characterized. In Dictyostelium discoideum and probably many other unicellular eukaryotes, Skp1 is modified by a pentasaccharide attached to a hydroxyproline near the C-terminus. This modification has previously been shown to be important for oxygen-sensing during Dictyostelium development and is mediated by the activities of a cytoplasmic HIF-alpha type prolyl 4-hydroxylase and three sequentially acting glycosyltransferases. We have now characterized AgtA as the enzyme responsible for the addition of the final two galactose residues in α-linkages to the Skp1 core glycan. AgtA is unique among the pathway enzymes in the possession of non-catalytic WD40-repeat domains at its C-terminus in addition to its single N-terminal catalytic domain. Through the use of domain deletions, binding studies, and enzyme assays, we show that the WD40 domains confer a novel second-site binding interaction with Skp1 that promotes modification by catalytic activation rather than or in addition to simple recognition. Activation is inhibited by binding to a model F-box protein. The ability of overexpressed catalytically inactive AgtA to interfere with Skp1 hydroxylation and glycosylation and cause a novel developmental phenotype, and the novel phenotype of a double mutant of agtA-disruption with disruption of the earlier acting gnt1 α-GlсNac-transferase, confirm that AgtA regulates Skp1 by more than simple catalytic α-galactosylation.

(124) Development, Characterization and Application of Sialyltransferase for In Vitro Glycoengineering
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In vitro glycoengineering is an interesting tool to alter the glycopattern of therapeutic proteins, e.g. for use as reference material for in vitro or in vivo characterization of the respective glycan variants. Also large scale application is possible to provide novel protein therapeutics for human use. On this poster the development and analytical characterization of two variants of human α-2,6 sialyltransferase are described as well as their application for in vitro glycoengineering of therapeutic proteins. The two variants show different activity/ specificity, which enables to achieve different degrees of sialylation of the target protein. Furthermore, the application of the enzyme for in vitro glycoengineering of therapeutic proteins is touched briefly including some analytical data.
Mucin type O-glycosylation is initiated by a large family of UDP-GalNAc:polypeptide GalNAc transferases. Structurally, the ppGalNAc-T's consist of an N-terminal catalytic domain linked via a short flexible linker to a C-terminal ricin-like lectin domain. The roles and properties of both domains are not understood but are believed to have unique functions in site recognition. Our lab has been systematically analyzing ppGalNAc-T peptide and (glyco)peptide substrate specificity, by exploiting a series of novel random (glyco)peptide substrates designed to probe the functions of the catalytic and lectin domains (Gerken et al., J.Biol. Chem., 286, 14493 (2011)). Recently, we have reported that the presence of an N-or C-terminal placed Thr-O-GalNAc can be an important determinant of overall catalytic activity and specificity which differ between 3 transferase isoforms (Gerken et al., J.Biol.Chem., 288, 19900 (2013)). In our studies, we noticed variable UDP-H-GalNAc hydrolysis (i.e. transfer to water) vs. transfer to (glyco)peptide substrate depending on isof orm. For the non-glycopeptide preferring transferases (T2, T5 & T13) profiles showed high UDP-H-GalNAc transfer with little to no hydrolysis with their most preferred substrates while high UDP-H-GalNAc hydrolysis was observed for their less-preferred glycopeptide substrates. In contrast, the glycopeptide preferring transferases (T4, T7, T10 & T12) showed significant UDP-H-GalNAc hydrolysis even on their most preferred glycopeptide substrates while very little transfer and very high hydrolysis was observed with their less-preferred substrates. These studies suggest that substrate (glyco)peptide binding at the catalytic domain competes with transfer to water. This is consistent with the X-ray structural studies of ppGalNAc T1 and T2 which show a flexible loop closing over the active site upon peptide substrate binding (Fritz et al., PNAS,101,15307, (2004)), (Fritz et al., J. Biol. Chem., 281, 8613, (2006)). Transferases were generous gifts of the following labs: L. Tabak, NIH, H. Clausen, University of Copenhagen, K. Moreman, University of Georgia and D. Jarvis, University of Wyoming. Supported by NIH grant R01 CA078834 to TAG.

(125) Investigating the role of glycopeptide acceptor on the hydrolysis of UDP-GalNAc donor by the UDP-GalNAc: polypeptide GalNAc transferases (ppGalNAc T’s)

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Human beta-galactoside alpha-2,6-sialyltransferase I (ST6Gal-I) establishes the final glycosylation pattern of many glycoproteins by transferring a sialyl moiety to a terminal galactose. Complete sialylation of therapeutic immunoglobulins is essential for their anti-inflammatory activity and protein stability, but is difficult to achieve in vitro owing to the limited activity of ST6Gal-I towards some galactose acceptors. The crystal structures of human ST6Gal-I in complex with the product CMP and in complex with cytidine and phosphate was solved. These complexes allow for the rationalization of the inhibitory activity of cytosine-based nucleotides. ST6Gal-I adopts a variant of the canonical glycosyltransf erase A fold and differs from related sialyltransferases by several large insertions and deletions that determine its regiospecificity and substrate specificity. A large glycan from a symmetry mate localizes to the active site of ST6Gal-I in an orientation compatible with catalysis. The glycan binding mode can be generalized to any glycoprotein that is a substrate of ST6Gal-I.

(127) Enzymatic basis for N-glycan sialylation: structure of rat ST6GAL1 reveals conserved and unique features for glycan sialylation

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Complex carbohydrates attached to glycoproteins and glycolipids play critical roles in biological recognition, targeting, and modulation of functions of the corresponding glycoconjugates in animal systems, where they provide an additional level of biological "information content" in various physiological contexts. Terminal capping of glycan structures with negatively charged sialic acid residues has been shown to form or mask recognition sites for a variety of biological functions as well as being employed by numerous pathogens for species-specific or cell-specific tropisms. In mammalian systems sialic acid linkages are synthesized through the action of a conserved family of sialyltransferases (20 distinct gene products found exclusively in CAZY family GT29) that have unique linkage and acceptor specificities. Structural data is available for several bacterial sialyltransferases that are remotely related to the CAZY GT29 sialyltransferases and a single mammalian sialyltransferase, porcine ST3GAL1, that synthesizes alpha-2,3-sialic acid linkages on O-glycan structures. In an effort to understand the structural basis for sialyltransferase specificity, we have expressed and determined the structure of rat ST6GAL1, an enzyme that creates terminal alpha-2,6-sialic acid linkages on complex type N-glycans. The enzyme was produced as a GFP fusion protein by large-scale transient transfection of HEK293 cells, tag sequences were removed by TEV protease cleavage, and glycan heterogeneity was reduced by expression in an HEK2935 GnT1-cell line followed by endoglycosidase F digestion. Crystals of the purified protein diffraction to 2.4 Å and the structure was solved by SAD phasing using Se-methionine labeling of the enzyme in mammalian cells. The resulting structure has an overall fold that broadly resembles pig ST3GAL1, including
a CMP-sialic acid binding site assembled from conserved sialylmotif sequences. Significant differences in structure and disulfide bonding pattern are found outside the sialylmotif sequences, including differences in the residues directly interacting with CMP-sialic acid and the entire glycan acceptor site. Modeling and molecular dynamics simulations of donor and acceptor structures in the active site of ST6GAL1 reveals the structural basis for glycan acceptor specificity and linkage position as well as the framework for catalysis by mammalian sialyltransferases. Supported by NIH Grants RR-005351, GM103390 and U54 GM074958 from the NIH Protein Structure Initiative.

(128) Can we use Fucose analogs to modulate Notch signaling?-Analysis of donor substrate specificity of Pofut1-Hideyuki Takeuchi1, Esam Al-Shareef1, Aime Lopez Aguilar2, Hao Jiang2, Wesley Zandberg3, David Vocadlo3, Peng Wu2, Robert S. Haltiwanger1

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Protein O-fucosyltransferase 1 (Pofut1) transfers Fucose from GDP-L-Fucose to epidermal growth factor-like (EGF) repeats which contain the appropriate consensus sequence. O-Fucose can then be elongated by the Fringe family of β3-N-acetylgalactosaminyltransferases. The biological importance of Pofut1 and Fringe is particularly evident in that they regulate the Notch signaling pathway. Furthermore, recent studies have suggested that Pofut1 levels are elevated in several types of cancer, while decreases in Lunatic fringe levels contribute to breast cancers. Thus, identifying reagents capable of modulating Pofut1 or Fringe activity could lead to the development of therapeutics for these diseases. In this study, we analyzed the effect of several Fucose derivatives on the activity of Pofut1 in vitro and their incorporation into proteins that are known to be O-Fucosylated, such as Notch1 in cells. A variety of GDP-L-Fucose analogs were obtained and examined in vitro enzymatic assays with recombinant EGF repeats (containing O-Fucose consensus sequences) and overexpressed Pofut1 enzyme, and products were analyzed by nanoLC-MS/MS. Preparative reactions were also performed to generate sufficient quantities of EGF repeats modified with the Fucose derivatives. These modified EGF repeats were then utilized to test Lunatic fringe activity in vitro. To test whether the Fucose derivatives were incorporated into EGF repeats in cells, peracetylated forms of the derivatives were added to cells expressing a portion of mouse Notch1 with several known O-Fucosylation sites in the presence or absence of Lunatic fringe. Site-specific incorporation of the derivatives into predicted O-Fucosylation sites was analyzed by nanoLC-MS/MS analysis after digestion with proteases. These studies should reveal whether derivatization of Fucose modulates Pofut1 activity and/or affects Fringe-dependent elongation. Finally, we are examining the effects of these derivatives on Notch activity using a variety of cell-based and in vivo Notch signaling assays. This work was supported by NIH grant GM61126 (RSH).

(129) Mutational analyses of human glucosamine-6-phosphate N-acetyltransferase: identification of amino acid residues critical for acetyltransferase activity

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Glucosamine-6-phosphate N-acetyltransferase 1 (GNA1) catalyses the transfer of an acetyl group from acetyl-CoA to glucosamine-6-phosphate to form N-acetylglucosamine-6-phosphate, a major intermediate in the biosynthesis of UDP-GlcNAc. Previous studies identified conserved residues in human GNA1 as critical for catalysis and substrate affinity. However, the roles of residues located near the binding pocket of the acetyl-CoA donor substrate have not been established. Since N-butyryl-glucosamine has shown protective effects on bone and cartilage, we are also interested in engineering an enzyme that can transfer the butyryl group to form N-butyryl-glucosamine-6-phosphate. Several conserved and non-conserved residues were found to reside on the β5 strand near the acetyl-CoA binding pocket in human GNA1, including Leu155, Cys157 and Asn161, while Glu156 was shown to be essential for acceptor substrate binding. In this work, we showed that acetyl-CoA was much more effective than butyryl-CoA as a donor substrate. To improve the butyryl transfer activity, we produced single and double site-specific mutants potentially affecting the dimensions and properties of the acetyl-CoA binding pocket. A novel, sensitive fluorescence-based microtiter plate assay was used to screen mutants. While most mutants remained active, the Leu155/Asn161 double mutant showed loss of acetyl transfer activity but retained the ability to transfer the butyryl group. The alterations in activities and donor specificities of the mutants suggest important roles of amino acid residues in catalysis, and direct further strategies for the enzymatic synthesis of N-butyryl-glucosamine. This work was supported by a Strategic Project grant from the Natural Sciences and Engineering Research Council of Canada.

(130) Identification of a Novel Glycosyltransferase Family via a Conserved Domain Search Combined with Toxic Lectin Sensitivity Studies

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Our group has previously shown that the Galβ1,3Gal/ GalNAc specific lectin Marasmius oraeae agglutinin (MOA) is highly toxic to C. elegans due to its binding to a glycosphingolipid species, most likely an α1,3Gal attached to the core β4GalNAc (Wohlschlager et al., 2011). More recently we have studied MOA toxicity in Drosophila. In this species our genetic data indicates that MOA similarly targets an epitope that is attached to the core glycosphingolipid β4GalNAc. Together these lectin studies indicate that both the C. elegans and Drosophila genomes encode a novel α3Gal-transferase that is active against the glycolipid β4GalNAc. To identify novel candidate proteins for a α3GalT activity we carried out a sequence independent search to identify protein domains that are conserved in the Drosophila and C. elegans genomes. We then
excluded domains that had known function or were found on proteins that did not have a domain organization consistent with our expectations for a Golgi localized glycosyltransferase. This was followed by searches against Drosophila and C. elegans databases to exclude proteins that were not expressed in MOA binding tissues or cell lines or had reported phenotypes inconsistent with a role in glycosphingolipid biosynthesis. This combined bioinformatics approach resulted in the identification of a single protein family that met all our inferred and experimental criteria. This protein family is invertebrate specific and does not show sequence homology to any known glycosyltransferases, however, using the Phyre server (Kelly and Sternberg, 2009) to predict protein structure from amino acid sequence the candidate is predicted to have a nucleotide-diphospho-sugar transferase fold, compatible with a glycosyltransferase activity. We have analyzed genetically in Drosophila the sole representative of this protein family and find that flies with reduced expression of the gene show reduced MOA binding. We also find that the mutants interact phenotypically with the genes that encode the preceding enzymes in the glycosphingolipid pathway. These data are consistent with a role for this novel glycosyltransferase in generating the MOA epitope and modifying invertebrate glycosphingolipids. We will present further genetic and biochemical characterization of this novel enzyme family.

(131) Differential Conformational Effects of O-GalNAc and O-Man Glycan Modifications on alpha-Dystroglycan, and their Implications for Function
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Ramifications of protein glycosylation are often viewed from the perspective of interactions between the pendant glycans and other molecules, with less attention devoted to their relationship to the glycoprotein scaffold to which they are appended, and to the potential for conformational affects on the protein component. Both of these aspects can affect function. Although generally only one form of glycosylation is associated with a particular region of a glycoprotein, an important exception to this is observed in alpha-dystroglycan (αDG), where its mucin-like region has been found to be modified with both O-Man and O-GalNAc glycans, a feature likely true in other O-mannosylated proteins, and adding additional complexity. Although the physiological role of the O-GalNAc glycans on αDG is not fully understood, these modifications have been shown to induce a more stable and extended structure in mucins and this is likely relevant to the mechanical role of αDG in linking the cytoskeleton to the extracellular matrix. The O-Man glycans have been associated with functional interactions and molecular recognition, and defects in them associated with forms of muscular dystrophy, but their impact on the protein conformation has not been established. Differential conformational effects may impact the glycosylation process, with implications for disease, given the sequential temporal relationship of the initiation of the two forms. To elucidate the relative conformational factors that may affect the interplay of these modes of O-glycosylation, we undertook a structural NMR study of two forms of a glycopeptide from aDG, chemically synthesized, one modified with O-Man and one with O-GalNAc. From the results it was concluded that the O-Man modification had a minor impact on the molecular conformation, while the O-GalNAc effect was significant. Subsequently, extensive molecular dynamics simulations were undertaken on these structures to further understand contributions to the molecular features. The combined results offer new insights into the conformational effects of O-glycosylation.

(132) Investigating the Effects of Fringe Modification on Drosophila Notch Structure and Function
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The Notch signaling pathway is conserved across all metazoans and is essential in several different developmental processes. Notch is a large single-pass transmembrane receptor. In Drosophila, one of two ligands, Delta or Serrate, binds to Notch to activate downstream signaling events. The extracellular domain (ECD) of Drosophila Notch contains thirty-six tandem epidermal growth factor-like (EGF) repeats, many of which are predicted to be glycosylated. O-Fucosylation by O-fucosyltransferase 1 (Ofut1) is essential for Notch function, and elongation of O-fucose with a β3-linked GlcNAc by Fringe modulates Notch activity by increasing Notch-Delta binding while decreasing Notch-Serrate binding. The molecular mechanism by which Fringe modification alters ligand binding is unknown. EGF repeats 11-12 are necessary and sufficient for ligand binding and are established as the core ligand binding domain. However, other EGF repeats have also been shown to affect ligand binding. Twenty-four of the thirty-six EGF repeats are predicted to be O-fucosylated, including EGF repeat 12 in the ligand-binding domain. Glycoproteomic mass spectral site-mapping data suggest that O-fucose exists on predicted sites at high stoichiometry, but also that Fringe modifies some EGF repeats more efficiently than others. Thus, both O-fucose mono- and disaccharide glycoforms vary across the O-fucosylated EGF repeats of Notch. We hypothesize that glycosylation by Fringe on specific EGF repeats alters the structure, or possibly oligomerization status, of the Notch ECD, which changes the ability of Notch to bind preferentially to one ligand over the other. We are using electron microscopy to evaluate changes in conformation and blue native polyacrylamide gel electrophoresis to analyze oligomerization status. Preliminary electron microscopy data suggest that Fringe modification may cause slight conformational changes in the Notch ECD. Supported by NIH grant R01GM061126 and T32GM008468.
Molecular mechanisms for Fringe affects on mouse Notch1
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The extracellular domain (ECD) of Notch contains up to 36 tandem Epidermal Growth Factor-like (EGF) repeats, many of which contain the consensus sequence for modification by O-Fucose (C\(^2\) xxx(T/S)C). O-Fucosylation is essential for Notch function, and addition of GlcNAc to O-Fucose by the Fringe family of β3-N-acetylgalosaminyltransferases modulates Notch activity. In the fly system, a single Fringe enzyme modulates Notch activity by increasing signaling initiated by the ligand Delta and inhibiting signaling initiated by the other ligand, Serrate. Fringe modification appears to affect the binding of Notch to ligands, enhancing Notch-Delta binding but reducing Notch-Serrate binding. Although similar results have been reported using mammalian components, the increased number of receptors (four Notch proteins: Notch1-4), ligands (three Deltas: Delta-like 1, 3, and 4; two Serrate homologues: Jagged1 and 2) and Fringes (three Fringes, Lunatic (Lfng), Manic (Mfng) and Radical (Rfng)) reveals increased complexity. To better understand the molecular mechanism of Fringe action, we have sought to determine which of the 20 predicted O-Fucose sites on mouse Notch1 (mN1) are modified with O-Fucose, which are elongated by each Fringe, and which are required to mediate the biological effects of Fringe. We expressed and purified mN1 EGF 1-36 (majority of the ECD) with or without co-expression of the three Fringes from HEK 293T cells and used semi-quantitative mass spectral analysis developed by our lab for mapping Fringe modified sites. We show that most predicted O-Fucosylation sites are modified with O-Fucose monosaccharide efficiently and that elongation past O-Fucose by the three Fringes is site-specific but variable. We mutated individual O-Fucose sites that are modified by the Fringes and examined whether they were required for the biological effects of Fringe using cell-based Notch signaling assays and cell-based Notch-ligand binding assays. The results indicate that Fringe-mediated elongation at multiple sites contributes to enhanced Notch-Delta signaling, while modification at a smaller number of sites is required to inhibit Notch-Jagged signaling. Supported by NIH grant GM061126.

(134) Hydroxylation and Glycosylation of Skp1 Promote its Binding to Two Different F-box Proteins in the Social Amoeba Dictyostelium
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In many protists, the Skp1 modification pathway is an oxygen-sensor regulating development. In the social amoeba Dictyostelium, Skp1 is hydroxylated on a unique proline residue which can be sequentially modified to yield an O-linked pentasaccharide. Skp1 is an adapter in the Skp1/ Cullin1/F-box protein (SCF) family of E3 ubiquitin ligases, in which F-box proteins (FBPs) are substrates or substrate receptors that target specific proteins for polyubiquitination and subsequent proteasomal degradation. Co-immunoprecipitation from cells queried by Western blotting and Orbitrap/ mass spectrometry proteomics showed that, in addition, glycosylation promotes Skp1 interactions with the FBPs FbxD and FbxA, the first step in assembly of the SCF complex. These effects were confirmed by reverse co-immunoprecipitation from FLAG-FbxD expressing strains. To address if glycosylation dependence is a property of the proteins themselves, mg quantities of native Skp1 glycoforms were produced recombinantly. Binding studies with the model FBP Fbs1/ Fbg1/ Ocp1 showed modestly enhanced binding to modified Skp1 isoforms. Analytical gel filtration and chemical cross-linking revealed that early modifications modestly inhibited Skp1 self-association and that late modifications decreased its hydrodynamic volume, and circular dichroism revealed increased α-helical content and decreased β-sheet content of glycosylated Skp1 but no effect on temperature-dependent denaturation. Promoting the assembly of SCF complexes (which include >50 predicted FBPs) may affect the stability of proteins required for development and serve as a model for how Skp1 modification contributes to proliferation of the pathogen Toxoplasma gondii, the causative agent of human toxoplasmosis.

(135) Physiological Function of Free N-glycan involved in Protein Folding
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Introduction: We have found that free N-glycans (FNGs), some of which has been believed to be generated by endoplasmatic reticulum-associated degradation (ERAD), occur at μM concentration in growing or differentiating plant tissues, but the physiological functions still remained to be elucidated. As a part of study on functional analysis of FNGs, we have analyzed chaperone-like or protein-folding stimulating activity of FNGs. In this study, we used Jack Bean α-mannosidase (JB Man′ase), a glycoprotein consisted of two different subunit carrying both high-mannose (HM) and truncated type N-glycans, and a non-glycosylated protein (3mutwil) as model proteins. Methods: JB Man′ase was denatured with SDS and mercaptoethanol, and HM type N-glycan on JB Man′ase was released by Endo-H. Removal of the HM N-glycan from JB Man′ase was confirmed by ConA lectin blotting. JB Man′ase activity was measured using PNP-α-Man. The conformational changes in JB Man′ase were analyzed by Circular Dichroism and Fluorescence measurement. Chaperone-like function of FNGs involved in the refolding of non-glycoprotein, 3mutwil, was analyzed by NMR. Results and Discussion: Denatured JB Man′ase was refolded and recovered the glycosidase activity after incubation in a refolding buffer, while denatured following deglycosylated JB Man′ase was not able to recover the activity. This result suggests that the linked HM type N-glycans was able to induce the refolding
of denatured protein and the recovery of enzyme activity. Furthermore, we have found that an addition of the high-mannose type FNGs (GN1-type) induced the protein-refolding and rescued the enzyme activity of denatured / deglycosylated JB Man’ase up to a significant level. Addition of FNGs to denatured 3mutwil was also able to induce the protein-folding and the refolding state was confirmed by NMR. These results suggest that not only linked N-glycans but also free N-glycans have protein-refolding stimulating activity. We postulate, therefore, that if FNGs reside in a specific place where the folding of nascent or unfolded proteins proceed, these FNGs are able to be involved in protein folding.

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(136) Immunoglobulin G1 Fc domain motions: implications for Fc engineering
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Immunoglobulin G (IgG) is key to the adaptive immune system’s role in pathogen identification and destruction. The antigen-specific Fab domains bind to foreign targets and cluster the Fc regions of IgGs, gathering transmembrane Fcγ receptors at the plasma membrane and eliciting a localized, cellular-based host immune response. Not surprisingly, multiple signaling nodes within the cell tightly regulate the committal to a damaging, though protective, immune response. However, it is unclear whether IgG itself serves as a checkpoint, potentially with receptor affinity modulated through conformational sampling or post-translational modification. Here we report the crystallization and structure determination of the Fc portion of IgG1 from human serum, engineered to have homogeneous, galactose terminated, biantennary glycans. The structure shows a previously unseen conformation of Fc. This in turn provoked an extensive molecular dynamics (MD) investigation into the range of Fc conformations that can be sampled. Conformational variations involve primarily reorientation of the N-terminal Cγ2 domains in the dimeric structure. The structure and mobility of the N-glycans will be discussed. Two components were identified that influence Cγ2 domain motion. Simulations of Fc lacking either the hinge or Cγ2/ Cγ3 interface salt bridges showed greater motion amplitude than the wild-type; furthermore, an Fc containing both interface mutations and the hinge deletion exhibited yet greater motion suggesting both features contribute to conformational restriction and thus optimal binding to Fcγ receptors. These results are consistent with a model of evolutionarily optimized Fc domain conformational freedom that can be tuned to regulate immune response by processes such as posttranslational modification, but still limit spurious activation.

(137) Labeling IgG1-Fc and Fc-glycans for the conformation/dynamic studies by NMR
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The immunoglobulin G1(IgG1) Fragment crystallizable (Fc) elicits an immune response by engaging the cell-surface Fc gamma receptors (FcγR). The structure and composition of the Fc N-linked glycans modulates binding specificities of antibodies to these receptors. The Fc N-glycan is naturally heterogenous and compositional changes are linked to multiple diseases, likely through conformational changes of Fc and thus FcγR affinity. To better explain these functional variations, we aimed to label both the Fc and the Fc N-glycan and study their conformational changes by solution NMR spectroscopy. The results pertaining with the uniform as well as selective N-amino acid labeling of Fc expressed in HEK293F cells, uniform (13C2-Gal) or selective (13C2-Gal) terminal galactose labeling of Fc-glycans and their NMR analysis will be discussed. It was noted that the type and distribution of glycoforms produced by HEK293F cells were remarkably similar to Fc purified from human sera. For glycan labeling, the unlabeled monosaccharide units of recombinantly expressed Fc-glycans are enzymatically cleaved by glycosidases and labeled by adding UDP-13C15N13C2-Gal as a substrate precursor for U 2 enzymatically incorporating into the trimmed glycans by galactosyltransferase. The ratio of in vitro labeling of glycans is analyzed by mass spectrometry and further the conformational changes is analyzed by NMR spectroscopy.

(138) PMM1 Suppresses PMM2 Deficiency In Zebrafish
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The first-identified and most prevalent form of Congenital Disorder of Glycosylation, PMM2-CDG(Ia), is due to defective alleles at the locus for PMM2, which encodes the phosphomannomutase enzyme normally responsible for synthesis of mannose-1-P (M1P) and hence GDP-mannose. Identification of PMM2 defects led to the hypothesis that disease was caused by M1P deficiency, and consequently depletion of lipid-linked oligosaccharides (LLOs) needed for N-glycosylation. However, this hypothesis had never been rigorously tested in a valid PMM2-CDG animal model. Using a zebrafish PMM2-CDG model, we found that accumulation of the PMM2 substrate M6P triggers hydrolysis of LLO. Direct analytical measurements, however, unexpectedly failed to show depletion of M1P or GDP-mannose. We hypothesize that a second M1P biosynthetic route, normally silent, is activated in the PMM2-CDG disease background. The enzyme encoded by the related gene PMM1 has a catalytic activity in vitro similar to PMM2-encoded enzyme, but is thought to be unnecessary for M1P production in vivo. We found...
that overexpression of PMM1 mRNA in PMM2-depleted zebrafish corrects several behavioral and biochemical defects, including elevation of M6P and loss of LLO, without affecting PMM2 mRNA itself. Purified PMM1 enzyme hydrolyzes mannose-1,6-bisphosphate (M1,6BP; an enzymatic cofactor for both PMM1 and PMM2), to yield M1P and/or M6P (the actual ratio cannot be determined due to the enzyme's inherent mutase activity, Biochem J. 339:201). Unexpectedly M1,6BP was highly abundant in zebrafish, and its levels were reduced by overexpressing PMM1. We suggest that in PMM2-CDG zebrafish, endogenous PMM1 enzyme may be activated to convert M1,6BP to M1P, replacing PMM2-generated M1P and forming a bypass of PMM2 deficiency. Moreover, in PMM2-CDG zebrafish overexpressing PMM1, we speculate that M6P is consumed by an unknown process to help replenish M1,6BP. PMM2-CDG disease might thus be managed by inducing PMM1 expression in tissues where it is normally silent. Supported by NIGMS grants GM038545 and GM086524.

(139) Suppression of cytosolic and acidic peptide:N-glycanase (PNGase) in Arabidopsis thaliana

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In growing or developing plant tissues, two types of free N-glycans (FNGs), GN1 and GN2 type, are always produced, suggesting a possibility that these FNGs are involved in plant growth and differentiation. As a part of study to elucidate the physiological function of FNGs, we have already constructed a transgenic Arabidopsis thaliana plant, in which two ENGase genes are completely knocked-out. In the strain, any ENGase activity could not be found, and free N-glycan resulted in the plant had the GN2-type structure instead of GN1-type structures (MannGlcNAc1 change could not be detected [Kimura et al. 2011]. In this study, therefore, we tried to construct another transgenic Arabidopsis plants, in which PNGase genes are knocked-out to suppress the formation of FNGs completely. In A. thaliana, there are one cytosolic PNGase (cPNGase) gene (At5g49570) and two putative acidic PNGase (aPNGase) genes (At5g14920, At5g05480). Since it has been believed that the cytosolic PNGase release high-mannose type N-glycans, prior to the action of ENGase, from misfolded proteins in the cytosol, we prepared cPNGase-knocked-out A. thaliana. When the cytosolic PNGase gene was knocked out, FNGs were still found in stem from the cPNGase knockout plant and all of FNGs had GN2-type structure, indicating that ENGase, in the absence of cPNGase, released high-mannose type N-glycans from misfolded glycoproteins. Now, we are preparing a double knockout of aPNGase in Arabidopsis plants to compare the structure of FNGs with those of cPNGase knockout lines. We would like to discuss the physiological significance of aPNGase involved in plant differentiation based on the structures of FNGs found in the transgenic plants.

Reference

(140) Hexosamine Metabolism in PGM3-Deficient Patient Fibroblasts

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Phosphoglucomutase 3 (PGM3) is a member of hekose phosphate mutase family and is responsible for catalyzing the interconversion of GlcNAc-6-P and GlcNAc-1-P, which is a key step in the hexosamine biosynthetic pathway. Patients with mutations in PGM3 show a novel constellation of immunologic and neurologic impairments cutaneous vasculitis, myoclonus, and cognitive impairment, and they have now been identified as another type of Congenital Disorder of Glycosylation. Previously it was reported that even though deletion of mouse Pgm3 is embryonic lethal, hypomorphic Pgm3 alleles result in reduced PGM3 enzymatic activity and UDP-GlcNAc pools, leading to cell-specific changes in glycosylation. Here we report that fibroblasts from three PGM3-deficient patients also have reduced PGM3 activity and decreased UDP-GlcNAc, but not UDP-Glc. Importantly, GlcNAc supplements in the medium restore depleted UDP-GlcNAc as well as UDP-GalNAc compared to controls where GlcNAc supplementation does not change the levels. PGM1 and PGM3 in vitro activity was directly measured using GC/MS without relying on coupled enzyme method. PGM3 activity from cell lysate was significantly reduced. It is also known that PGM3 interconverts Glc-6-P and Glc-1-P, normally catalyzed byPGM1, which is a key step affecting glycolysis/ glycogenesis pathways. Preliminary results suggest PGM3 mutation did not affect such interconversion, suggesting these processes are presumably normal. Our finding of improvement of UDP-GlcNAc levels by adding GlcNAc suggests that GlcNAc/GlcN supplementation would be a potential treatment for these PGM3-deficient patients. [Supported by R01DK55615 and The Rocket Fund]

(141) Mannose metabolism in MPI null mouse embryonic fibroblasts

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Phosphomannose isomerase (MPI) is the only known enzyme that interconverts Fru-6-P and Man-6-P to supply glucose-derived mannose from glucose for N-glycosylation. Hypomorphic mutations in MPI cause a genetic disease, CDG-Ib, due to aberrant N-glycosylation, but providing Mannose supplements normalizes N-glycosylation and symptoms. However, mice carrying a null mutation in Mpi accumulate excessive Man-6-P since it cannot be catabolized. Studies in embryonic fibroblasts (MEF) from these mice showed that excess Man-6-P inhibits glycolysis. To study the fate of accumulated intracellular Man-6-P further, we established: (1) a method for quantification/analysis of sugar phosphates and other metabolites by GC/MS, and (2) the origin and/or fate of those metabolites using a combination of stable isotopes, U-13C-Man/U-13C-Glc, as tracers. U-13C-Man labeling of Mpi-/MEF, revealed that accumulated Man-6-P was transformed into other sugar phosphates such as Man-1-P, (mannitol-1-P), Man and Mannitol. Surprisingly, it was also
mannosyltransferases are undergoing. GDP-Glc and GDP-GlcNH. Applying these molecules in studying the synthesis of GDP-Man and analogues, as well as GDP-Tal, GDP-GlcNAc was not successful. (33%), and low yield of GDP-Man4N (16%). However, the synthesis of GDP-ManNAc and GDP-GlcNH2 (80%) from corresponding monosaccharides. In addition, it gave moderate yield of GDP-Tal (47%) and GDP-ManF (84%), GDP-Glc (72%), GDP-2-deoxyGlc (76%), and GDP-GlcNH2 (80%) from corresponding monosaccharides. 81%), GDP-Man (94%), GDP-ManNH 75%), GDP-ManN 81%), GDP-ManF (84%), GDP-Glc (72%), GDP-2-deoxyGlc (76%), and GDP-GlcNH2 (80%) from corresponding monosaccharides. In addition, it gave moderate yield of GDP-Tal (47%) and GDP-Man4N (16%). However, the synthesis of GDP-ManNAc and GDP-GlcNAc was not successful. (33%), and low yield of GDP-GlcN. In summary, we have developed an efficient strategy for the synthesis of GDP-Man and analogues, as well as GDP-Tal, GDP-Glc and GDP-GlcNH. Applying these molecules in studying mannosyltransferases are undergoing.

(142) One-pot chemo-enzymatic synthesis of GDP-Man and analogues
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Sugar nucleotides and analogues are valuable substrates and probes to investigate glycosyltransferases. Preparation of such molecules is therefore of great interest and has been an active research field. In the past several years, our group had developed facile and efficient approaches to obtain GDP-A-Fuc, UDP-GlcNAc, UDP-GalNAc and their analogues. Herein, we describe the preparation of another common sugar nucleotide—GDP-Man—and analogues via a one-pot multi-enzyme strategy starting from corresponding monosaccharides. Among guanosine 5'-diphosphate (GDP)-activated sugars, GDP-Man is essential for the biosynthesis of eukaryotic glycans, glycosylphosphoinositol (GPI) anchors, and bacterial cell-surface polysaccharides. GDP-Man is also a fundamental metabolic intermediate for the biosynthesis of many other natural GDP-sugars, including GDP-mannuronic acid GDP-A-Fuc, GDP-Rha et al. Other GDP-sugars, such as GDP-glucose (GDP-Glc) and GDP-glucosamine (GDP-GlcNH2) are key intermediates in the biosynthesis of β1,4-glucans, glucosylglycerate, and legionaminic acid-containing glycoconjugates. In our strategy, three enzymes were used in one-pot to synthesis GDP-Man and analogues. The first enzyme was NahK, which catalyzed the formation of monosaccharide 1-phosphates. The second enzyme was PFManC, which catalyzed the reversible formation of GDP-sugar and pyrophosphate from monosaccharide 1-phosphate and guanosine triphosphate (GTP). The last enzyme was an inorganic pyrophosphatase cloned from E. coli (EcPPA). It drives the reaction towards the formation of GDP-sugars by hydrolyzing the pyrophosphate by-product. The system was very efficient in synthesizing GDP-Man (94%), GDP-ManNH (75%), GDP-ManN (81%), GDP-ManF (84%), GDP-Glc (72%), GDP-2-deoxyGlc (76%), and GDP-GlcNH2 (80%) from corresponding monosaccharides. In addition, it gave moderate yield of GDP-Tal (47%) and GDP-Man4N (16%). However, the synthesis of GDP-ManNAc and GDP-GlcNAc was not successful. (33%), and low yield of GDP-GlcN. In summary, we have developed an efficient strategy for the synthesis of GDP-Man and analogues, as well as GDP-Tal, GDP-Glc and GDP-GlcNH. Applying these molecules in studying mannosyltransferases are undergoing.

(143) Attenuation of fibroblast growth factor signaling by poly-N-acetyllactosamine type glycans implicated to human sperm motility
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Previous studies, including our own (Hatakeyama et al., J. Urol. 180, 767-71, 2008), suggested that mature human sperm cells are densely covered by carbohydrates. While we attempted to remove glycans from living human spermatozoa using glycosidases, we found human sperm cells treated with endo-beta-galactosidase (EBG), which specifically hydrolyzes poly-N-acetyllactosamine type glycans (polyLacs), moved more rapidly than untreated sperm. Mass spectrometry analysis revealed that sperm-associated polyLacs are linear, terminally siaylated and sulphated, and heavily fucosylated, consistent with Lewis Y antigen. Immunochemistry of epididymis using an anti-Lewis Y antibody before and after EBG treatment suggested that polyLacs carrying the Lewis Y epitope are synthesized in epididymal epithelia and secreted to seminal fluid. EBG-treated sperm elevated cAMP levels and calcium influx, indicating activation of fibroblast growth factor (FGF) signalling. Plate assay and glycan array demonstrated positive binding between FGFs and polyLacs. Furthermore, heparin did not inhibit polyLac binding to FGFs, suggesting that FGF has distinct binding sites each for heparin and polyLacs. Since both FGFs and polyLacs are expressed widely in mammalian tissues and in many cell types, we tested effect of polyLacs on FGF signaling using human kidney epithelial HEK293T cells, which showed down-regulation of tyrosine phosphorylation of FGF receptor. These results suggest that polyLacs may regulate FGF signalling in a variety of cell types and that sperm cells employ a conserved mechanism requiring highly concentrated polyLacs to regulate motility. This study was supported by Challenging Exploratory Research 24659726 from the Japan Society for the Promotion of Science (KS), National Institutes of Health Grants CA33859 (MF) and EY014620 (AT), and the Taiwan National Core Facility Program for Biotechnology, NSC grant 100-2325-B-001-029 (KHK). We thank Drs. David F. Smith and Jamie Heimborg-Molinaro for glycan array provided as core H by the Consortium for Functional Glycomics supported by NIH grants GM62116 and GM098791.

(144) Regulated N-Glycosylation in Pancreatic Beta Cells: An Evolutionary Adaptation in Response to Starvation?
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Obesity is a high risk factor for the onset of Type 2 diabetes (T2D). Recent discoveries by this laboratory have reported that protein
N-glycosylation plays a major role in the pathogenesis of obesity-associated diabetes. The GnT-4a glycosyltransferase, encoded by the MGAT4A gene in humans, produces a N-glycan linkage essential for pancreatic beta cell function by increasing the half-lives of glucose transporters on the beta cell surface. This regulatory mechanism exists in both humans and mice and is disrupted in humans with T2D and in the obesity-associated mouse model of T2D. In both species, the transcription factors required for Mgat4a expression in beta cells are down-regulated with the internalization and degradation of glucose transporters, resulting in loss of glucose-stimulated insulin secretion -a hallmark of human T2D. Enforced expression of beta cell-specific GnT-4a protein glycosylation and glucose transporter expression was found to protect animals from the onset of obesity-associated diabetes. More recently published studies have provided a computational model of glucose transport indicating further how a defect in pancreatic beta cell glucose transporter glycosylation and expression may lead to T2D. Although relevant to the pathogenesis of diabetes, it remains unclear why species have evolved and conserved a mechanism that renders pancreatic beta cells unable to secrete insulin in response to blood glucose. It has been reported by others that fasting disables glucose-stimulated insulin secretion in humans. We hypothesized that GnT-4a glycosylation may be diminished during times of fasting and starvation as an advantageous response to maintain blood glucose levels. We measured blood glucose and insulin abundance in MGAT4A transgenic mice during fasting. MGAT4A transgenic mice had lower blood glucose with higher insulin levels in circulation, compared with wild-type littermates. Our findings indicate that down-regulation of GnT-4a glycosylation in pancreatic beta cells during periods of nutrient deprivation allows animals to maintain higher blood glucose levels, thus likely supporting active foraging behaviors for longer periods and promoting survival. This same potential mechanism of thirst and survival is induced in obesity and implicated in the origin of T2D at current epidemic levels in the human population.

Caspase-3 is a member of the cysteine-aspartic acid protease (caspase) family and is a downstream of apoptosis signaling. Furthermore, the activated caspase-3 degraded Nanog protein in 3-O-HS overexpressed mESCs. These data showed that the 3-O-HS was required for differentiation of mESCs via caspase cascade. Therefore we assumed that Fas signal, major signal of apoptosis, was up-stream of caspase-3 activation during differentiation. Fas was transferred to lipid rafts on the cell surface by up-regulation of the 3-O-HS and then Fas signal was activated. The transfer of Fas to lipid rafts was inhibited by treatment with brefeldinA, which inhibits the transport of proteins from ER to Golgi. It indicated that Fas was transferred by increased 3-O-HS through the Golgi apparatus. Moreover the 3-O-HS interacted with a region that included the heparin-binding domain (KLRRRVH) of Fas. Reduced self-renewal ability in cells overexpressing 3OST-5 resulted from the degradation of Nanog by activated caspase-3, which is downstream of Fas signal, and was rescued by the inhibition of Fas signaling. Surprisingly, increased 3-O-HS did not affect FGF4/ ERK signaling, which demonstrated that Fas signaling via 3-O-HS induced the differentiation independently of FGF4/ ERK signaling. This finding showed that the activation of Fas signal mediated by the increase in the 3-O-HS promotes various types of differentiation in mESCs. And Fas signaling contributes to a novel mechanism of the differentiation after FGF4/ ERK signaling.

(145) Fas signaling via 3-O-sulfated heparan sulfate is involved in a novel mechanism of the differentiation of mouse ES cells

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Mouse embryonic stem cells (mESCs) are regulated by several extrinsic signals. FGF4/ ERK signaling is a key signaling inducing the differentiation of mESC. However, other mechanisms of the differentiation remain unclear. Sulfated heparan sulfate (HS) structures are significant for the bindings between HS and ligands such as FGFs. Here we screened HS structure involved in the differentiation of mESCs by overexpression of sulfotransferases and found that 3-O-sulfated HS structures (3-O-HS) synthesized by HS 3-O-sulfotransferase-5 (3OST-5) contributed to mESC differentiation into primitive endoderm and epiblast, including transition to epiblast stem cell (EpiSC). To elucidate the mechanism of the differentiation via 3-O-HS, we performed the overexpression of 3OST-5 in mESCs. Increased 3-O-HS reduced the self-renewal, caused apoptosis-like morphology, and induced the activation of caspase-3.

(146) Ganglioside-interacting proteins in nerve cells: Linking gangliosides to glutamate receptor expression

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Gangliosides – sialylated glycosphingolipids – are expressed on the plasma membranes of all vertebrate cells, but are enriched in nerve cells. Rare human disorders of ganglioside biosynthesis and mouse genetic models implicate altered ganglioside expression in neurological disorders, including severe seizures. The molecular mechanisms that connect ganglioside expression to seizures have not been established. Using an unbiased affinity-based screening method, we identified nerve cell surface proteins that bind differentially to GT1b, a major nerve cell ganglioside, compared to GM1, a major myelin ganglioside. Gangliosides were ozonolyzed to generate a unique aldehyde at the C4 carbon of sphingosine. The products were covalently bound to amine beads via reductive amination. Primary cerebellar granule neurons from rat pups were cultured for 12 days to allow a mat of axons to grow, and then their cell surface proteins were tagged with sulfo-NHS-SS-biotin, solubilized with detergents, and collected by streptavidin chromatography. Surface proteins were recovered by disulfide reduction, and equal portions applied to GT1b- and GM1-derivatized beads. After washing, ganglioside-bound proteins were separately eluted, differentially mass tagged, remixed, and subjected to mass spectrometric interaction analyses (iTRAQ) to reveal quantitative differences between GT1b- and GM1-bound proteins. A list of proteins was obtained (<1% false discovery rate) using Proteome Discoverer with Mascot and Sequest software to search the RefSeq rat protein database. Rigorous Bonferroni statistical analyses of differential GT1b/ GM1

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binding resulted in six GT1b-selective binding proteins (range 4-7 fold enhanced GT1b binding). One of these, Thorase (ATPase family AAA domain-containing protein 1), regulates the cell surface expression of the AMPA subtype of glutamate receptors. AMPA receptors mediate the majority of fast excitatory transmission in the human brain and are key mediators of seizures. Thorase functionally regulates the expression of AMPA receptors in an ATP-dependent manner. Secondary Western blot analyses demonstrated that GT1b selectively binds Thorase in an ATP-dependent manner. Comparison of the phenotypes of ganglioside-and Thorase-knockout mice suggests the potential of related functional pathways. Our data support a ganglioside-mediated AMPA receptor expression pathway that may regulate excitatory neurotransmission and whose dysregulation may result in seizure disorders. This work was supported by NIH grant NS037096.

(147) Ashwell-Morell Receptor Function Increases Host Susceptibility to Salmonella Typhimurium
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Salmonella enterica is a Gram-negative bacterium that causes a greater human disease burden than any other foodborne pathogen in the United States, and is responsible for up to 50% of bacteremias in young children in developing countries. Salmonella enterica Typhimurium (ST) is the reference pathogen of this species and interacts with the host in ways not fully understood to provoke inflammation, thereby contributing to the tissue damage and lethality of sepsis. Unlike the previously described protective role of the Ashwell-Morell receptor (AMR) in sepsis due to Streptococcus pneumoniae infection (Grewal et al., 2008; 2013), we have recently discovered that AMR function is deleterious to the host during sepsis caused by Salmonella Typhimurium (ST). We have further observed a similar result in studies of host outcomes following infection with ‘hypervirulent’ Salmonella Choleraesuis (SC), a serovar recently discovered to be among the most virulent microbes encountered of this species. These findings of altered host susceptibility are associated with AMR-dependent modulation of the abundance of specific glycoproteins. This includes host alkaline phosphatase (AP) isozymes that the AMR normally maintains within a narrow window of physiologic activity in blood circulation, likely to minimize the potential for metabolic dysfunction in bone development and homeostasis. However, AP also plays an important role in detoxifying the lipopolysaccharide (LPS) of Gram-negative pathogens (e.g., Salmonella) de-phosphorylating LPS and thereby disabling the ability of LPS to activate inflammatory signalling by the Toll-like receptors of the innate immune system. Our ongoing studies are determining the mechanistic linkages between AMR regulation of AP activities and host susceptibility to sepsis caused by ST infection. In related studies, we are focusing further on this metabolic axis to investigate AMR involvement and AP regulation in the onset of gastrointestinal inflammatory disease brought about by excessive AMR clearance of AP isozymes due to insufficient AP sialylation. These unexpected findings imply that the AMR is positioned at a crossroads of competing natural forces composed of pathogen-host interactions that have locked the AMR into both advantageous and disadvantageous host functions in the pathogenesis of sepsis.

(148) The role of galectin-3 in bone
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Despite having normal bone mass, patients with type 2 diabetes mellitus are at an increased risk of fracture. The accumulation of advanced glycation end-products (AGE) can alter cellular function and disrupt tissue homeostasis through AGE receptors. With the growing rise in type 2 diabetes, further work needs to be done to understand the function of AGE receptors, in order to develop therapeutics to prevent AGE induced tissue damage. The AGE receptor, AGE-R3 / galectin-3 (Gal3), is a dual AGE-and glycan-binding protein expressed in bone during normal development. Gal3 has known roles in collagen adhesion, Akt signaling, β-catenin stabilization, and AGE degradation, all of which are critical for bone cell function and bone homeostasis. However, it is not clear how Gal3 regulates these processes in bone, and whether these functions are perturbed in diabetes when Gal3 may get diverted into the AGE degradation pathway. Currently we are exploring the role of Gal3 in bone cell differentiation as well as the composition of bones isolated from Gal3 knock-out mice. Our preliminary data suggests that while loss of Gal3 does not greatly alter the gross morphology of bones in mice, these bones have altered bone matrix composition likely due to altered bone cell activity.

(149) Reducing Macrophage Proteoglycan Sulfation Exacerbates Diet-Induced Obesity via Type-I Interferon Signaling
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Obesity is a major contributor to cardiovascular disease related mortality and is setting-back progress made by risk-factor prevention. Diet-induced obesity has become a worldwide epidemic and is associated with a chronic low-grade inflammation, called metabolic inflammation, which is considered to be the driving force for obesity-related metabolic consequences such as Type-2 diabetes and atherosclerosis. A key mechanism underlying obesity-induced inflammation is accumulation of increased numbers of pro-inflammatory macrophages in obese adipose tissue. In order to examine the role of macrophage heparan sulfate proteoglycans in diet-induced obesity, we inactivated the biosynthetic gene GlcNAc N-deacetylase/ N-sulfotransferase 1 (Ndst1) selectively in

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macrophages by crossing Ndst1\textsuperscript{fl/fl} mice with LysMCre\textsuperscript{+} mice. Ndst1 inactivation reduced the overall sulfation of HSPG in macrophages by 30%. In spite of this modest change in heparan sulfate, Ndst1 LysMCre\textsuperscript{+} mice on a high fat diet had excessive body weight gain compared to control mice. Aged-matched mice on a chow diet did not show any differences in body weight gain. The increased fat content in liver, white adipose tissue and brown adipose tissue seen in Ndst1 LysMCre\textsuperscript{+} mice on the high fat diet was associated with increased macrophage infiltration and CCL2 expression, hallmarks for advanced metabolic inflammation. Glucose and insulin tolerance tests confirmed that Ndst1\textsuperscript{fl/fl} LysMCre\textsuperscript{+} mice had reduced insulin sensitivity. Microarray analysis of bone marrow derived macrophages from Ndst1\textsuperscript{fl/fl} LysMCre\textsuperscript{+} mice showed significantly increased expression of inflammatory genes such as CCL5, CCL7, CCL8 and TNF-alpha. Motif analysis of promoters of up-regulated genes revealed increased Type-I interferon (IFN) signaling in mutant macrophages. Also IFN-beta induced STAT1 phosphorylation was elevated in Ndst1\textsuperscript{fl/fl} LysMCre\textsuperscript{+} macrophages. We show that IFN-beta interacts with macrophage heparan sulfate, suggesting that macrophage proteoglycans control inflammation by maintaining Type-I interferon receptor in a quiescent state through sequestration of IFN-beta. Altogether our data imply that differences in macrophage heparan sulfation can possibly predict and determine the outcome of metabolic inflammation in diet-induced obesity.

(150) Reduction of Lectin Valency Drastically Changes Glycolipid Dynamics in Membranes but Not Surface Avidity
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Multivalency is proposed to play a role in the strong avidity of lectins for glycosylated cell surfaces, and also in their ability to affect membrane dynamics by clustering glycosphingolipids (Arnaud et al. 2013a). RSL, that has been chosen as the paradigm for designing neolectins, is a fucose-binding lectin from the bacterium \textit{Ralstonia solanacearum} that adopts a \textbeta-propeller fold formed by trimer association and presenting six binding sites. Alteration of the symmetry of the \textbeta-propeller architecture is used to produce neoRSLs (nRSLs) with controlled valency in order to improve diversity in the development of technological tools and to understand the endocytosis mechanism. After identification of key amino acids by molecular dynamics calculations, two mutants with reduced valency were produced. Isothermal titration calorimetry confirmed the loss of three high avidity binding sites for both mutants (Arnaud et al. 2013b). Crystal structures indicated that residual low avidity binding occurred in W76A, but not in R17A. The trivalent R17A mutant presented unchanged avidity towards fucosylated surfaces, when compared to hexavalent RSL. However, R17A is not able anymore to induce formation of membrane invaginations, indicating the crucial role of number of binding site number for clustering of glycolipids. Further modifications of the number and topology of binding sites in neoRSL was possible by special design of the neolectin gene. Correlation appeared between the invagination capacity and the number of binding sites, but also their topology and distances.

References

(151) Pentavalent siglecs: high avidity tools for detection of siglec counter-receptors
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Siglecs (sialic-acid-binding immunoglobulin-like lectins) are expressed primarily on subsets of immune system cells to regulate the functions of cells in the innate and adaptive immune systems through sialic acid dependent glycan recognition. Exemplary are human Siglec-8 and its mouse counterpart Siglec-F, expressed on eosinophils. Ligating with anti-siglec antibody or multivalent glycan counter-receptors results in eosinophil apoptosis. Likewise, ligating Siglec-9 or its mouse counterpart Siglec-E on neutrophils results in their apoptosis. Presumably, when siglec-expressing inflammatory cells encounter polyvalent sialoglycans (counter-receptors) on target tissues, the ongoing response is damped. Chimeric siglecs are used to explore endogenous counter-receptors. Because the monovalent site affinity of lectins is often modest, polyvalent forms are needed as efficient tools. Using the natural penta-merizing polypeptide domain from cartilage oligomeric matrix protein (COMP), we engineered pentavalent tagged siglecs and demonstrate their enhanced affinity in well-defined sialoglycan recognition studies and utility in endogenous counter-receptor detection. Expression plasmids were designed to express the amino-terminal extracellular domains of Siglec-7, -8, -9, -E, and -F followed by a BAP biotinylation site, a Factor Xa cleavage site, domains 3 & 4 of CD4 (as spacer), the COMP pentamerization domain, and a 6-His C-terminal tag. The COMP-siglec constructs were transiently expressed using an HEK293 suspension culture system suited for high-yield production of recombinant proteins, providing up to 50 mg/L of chimeric product. Gel filtration of purified COMP-siglecs revealed spontaneous multimerization, whereas SDS-PAGE revealed a well-defined monomeric product. Binding to defined glycan arrays revealed expected specificities with enhanced avidity. Compared with the rapid (minutes) off rate of the comparable siglec-Fc chimera, the siglec-COMP had a binding half-life of half a day. The resulting binding tools were capable of detecting sialic acid dependent counter-receptors on Western blots and by immunohistochemistry. Spontaneously
Lectins play an important role in host-pathogen interactions, where they help pathogens in adhesion to sugar moieties presented on host cells. Hence, lectins from pathogenic microorganisms and understanding of molecular basis of their binding modes have been intensively studied. Lectins belonging to the PA-III lectin family show a unique binding mode among bacterial lectins. They bind sugars through two calcium cations with very high affinity (Kd in micromolar range). Although these lectins are orthologues with significant sequential and structural similarities and they can bind similar saccharides, they strongly differ in specificities. PA-III from *Pseudomonas aeruginosa* [Mitchell et al. 2002] and CV-III from *Chromobacterium violaceum* [Pokorna et al. 2006] prefer L-fucose (and its derivatives) while RS-III from *Ralstonia solanacearum* [Kostlanova et al. 2005] and BC2L-A from *Burgholderia cenocepacia* [Lameignerev et al. 2008] prefer D-mannose (and its derivatives). Three main amino acids responsible for sugar specificity form so-called "specificity binding loop". The previous study on PA-III showed that the first amino acid from this loop (serine 22) is responsible for lectin specificity to fucose and its substitution to alanine (present in RS-III and BC2L-A) lead to overturning of specificity towards D-mannose [Adam et al. 2007]. We prepared three mutant proteins with one amino acid substitution in the mentioned position (CV-III_S22A, RS-III_A22S and BC2L-A_A29S) via site-directed mutagenesis. Isothermal titration microcalorimetry and crystallography served for study of molecular basis of binding modes of all prepared mutants. In all cases the substitutions led to changed specificity between L-fucose and D-mannose. In addition, the BC2L-A mutant displays unusual thermodynamic profile that is not common in protein/carbohydrate interactions. This work has been supported by Czech Science Foundation (P207/11/1815) and CEITEC – Central European Institute of Technology with research infrastructure supported by the project CZ.1.05/1.1.00/02.0068 from European Regional Development Fund.

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(152) Study of changes in specificity and affinity of lectins from PA-III lectin family
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Lectins play an important role in host-pathogen interactions, where they help pathogens in adhesion to sugar moieties presented on host cells. Hence, lectins from pathogenic microorganisms and understanding of molecular basis of their binding modes have been intensively studied. Lectins belonging to the PA-III lectin family show a unique binding mode among bacterial lectins. They bind sugars through two calcium cations with very high affinity (Kd in micromolar range). Although these lectins are orthologues with significant sequential and structural similarities and they can bind similar saccharides, they strongly differ in specificities. PA-III from *Pseudomonas aeruginosa* [Mitchell et al. 2002] and CV-III from *Chromobacterium violaceum* [Pokorna et al. 2006] prefer L-fucose (and its derivatives) while RS-III from *Ralstonia solanacearum* [Kostlanova et al. 2005] and BC2L-A from *Burgholderia cenocepacia* [Lameignerev et al. 2008] prefer D-mannose (and its derivatives). Three main amino acids responsible for sugar specificity form so-called "specificity binding loop". The previous study on PA-III showed that the first amino acid from this loop (serine 22) is responsible for lectin specificity to fucose and its substitution to alanine (present in RS-III and BC2L-A) lead to overturning of specificity towards D-mannose [Adam et al. 2007]. We prepared three mutant proteins with one amino acid substitution in the mentioned position (CV-III_S22A, RS-III_A22S and BC2L-A_A29S) via site-directed mutagenesis. Isothermal titration microcalorimetry and crystallography served for study of molecular basis of binding modes of all prepared mutants. In all cases the substitutions led to changed specificity between L-fucose and D-mannose. In addition, the BC2L-A mutant displays unusual thermodynamic profile that is not common in protein/carbohydrate interactions. This work has been supported by Czech Science Foundation (P207/11/1815) and CEITEC – Central European Institute of Technology with research infrastructure supported by the project CZ.1.05/1.1.00/02.0068 from European Regional Development Fund.

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(153) Recognition of a Mycobacterial Glycolipid by the Macrophage Receptor Mincle
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Trehalose dimycolate, an unusual glycolipid on the surface of *Mycobacterium tuberculosis* and *Mycobacterium bovis*, can initiate responses that cause either neutralization or protection of these pathogens. The macrophage receptor mincle appears to be a key mediator of these responses. Mincle, a transmembrane receptor containing an extracellular C-type carbohydrate-recognition domain (CRD), stimulates the Syk-CARD pathway by interacting with the common FeR1γ subunit. Structural analysis, mutagenesis, and ligand-binding studies with glycolipid analogs have been employed to define the mechanism of bovine mincle binding to trehalose dimycolate. The CRD contains an unusual extended binding site that encompasses both sugars in the Glc1-1Glcζ headgroup and also interacts with at least one of the attached acyl chains. One glucose residue occupies the canonical primary binding site seen in C-type CRDs, in which the 3- and 4-hydroxyl groups are ligated to a bound Ca. The second glucose residue also contacts the surface of the CRD through a set of hydrogen bonds. The resulting secondary binding site leads to 36-fold tighter binding of trehalose compared to glucose. A hydrophobic groove adjacent to the 6-hydroxyl group of the glucose residue in the primary sugar-binding site interacts with acyl chains attached to this hydroxyl group. Attachment of octanoic acid to one of the sugar residues in trehalose increases the affinity for mincle by 52-fold. Such a hydrophobic groove is not seen in other C-type CRDs. The results suggest that small molecule analogues of trehalose dimycolate can be used for targeted disruption of the interaction between the macrophages and mycobacteria. In addition to providing potential routes to preventing mycobacteria from successfully colonizing macrophages, the results could provide a basis for designing adjuvants that stimulate a response to immunization in the same way that mycobacteria do. Such synthetic adjuvants would have potential use in vaccine development.

(154) Common Polymorphisms in Human Langerin Change Specificity for Glycan Ligands
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Langerin, a C-type lectin on Langerhans cells, mediates carbohydrate-dependent uptake of pathogens in the first step of antigen presentation to the adaptive immune system. Langerin binds a diverse range of endogenous and pathogen cell surface carbohydrates, including high mannose structures, fucosylated blood group antigens and unusually for a “mannose-type” C-type lectin, glycans...
with terminal 6-sulfated galactose. Salt bridges between the sulfate group and two lysine residues appear to compensate for the non-optimal binding of galactose at the primary Ca₂⁺ site. Mutagenesis and quantitative binding assays indicate that removal of either lysine side chain results in a significant decrease in affinity for 6SO₄⁻Gal.

A single nucleotide polymorphism (SNP) in human langerin results in change of one of these lysine residues, Lys313, to Ile. Glycan array screening with the Ile313 form of langerin shows that this amino acid change abolishes binding to oligosaccharides with terminal 6SO₄⁻Gal and enhances binding to oligosaccharides with terminal GlcNAc residues. Genomic analysis shows that the Ile313 SNP is linked to another SNP which results in the change Asn288Asp. If Ile is present at position 313, residue 288 is always Asp. Residue 288 is near the principal Ca²⁺ site to which the sugar binds and our previous analysis shows that the Asn288Asp change reduces sugar-binding affinity. On the glycan array, langerin with Asp288 and Ile313 shows similar specificity for glycan ligands to the Ile313 form, with no binding to 6SO₄⁻Gal-terminated ligands and increased binding to GlcNAc-terminated structures, but overall decreased binding to glycans. The results indicate that the change of Lys to Ile at position 313 results in altered specificity for glycan ligands, while the Asn288Asp change decreases the overall affinity for glycans. Structural analysis of the carbohydrate-recognition domains of the variant langerin forms in complex with GlcNAc-terminated glycans shows the basis for the enhanced binding to GlcNAc, with Ile313 packing against the N-acetyl group. Altered langerin function in individuals with the linked Asn288Asp and Lys313Ile polymorphisms may affect susceptibility to infection by micro-organisms.

(155) Global comparisons of lectin-glycan interactions using a database of analyzed glycan array data

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Lectin-glycan interactions have critical functions in multiple normal and pathological processes, but the binding partners and functions for many glycans and lectins are not known. An important step in better understanding glycan-lectin biology is to enable systematic quantification and analysis of the interactions. Glycan arrays can provide the experimental information for such analyses, and the thousands of glycan array datasets available through the Consortium for Functional Glycomics (CFG) provide the opportunity to extend the analyses to a broad scale. We developed software, based on our previously-described Motif Segregation algorithm, for the automated analysis of glycan array data, and we analyzed the entire storehouse of 2,883 datasets from the CFG. We mined the resulting database to make comparisons of specificities across multiple lectins and comparisons between glycans in their lectin receptors. Of the lectins in the database, viral lectins were the most different from other organism types, with specificities nearly always restricted to sialic acids, and mammalian lectins had the most diverse range of specificities. Certain mammalian lectins were unique in their specificities for sulphated glycans. Simple modifications to a lactosamine core structure radically altered the type of lectins that were highly specific for the glycan. Unmodified lactosamine was specifically recognized by plant, fungal, viral, and mammalian lectins; sialylation shifted the binding mainly to viral lectins; and sulphation resulted in mainly mammalian lectins with the highest specificities. We anticipate value for this analysis program and database in fundamental glycobiology studies, detailed analyses of lectin specificities, and practical applications in translational research.

(156) Structural Characterization of the DC-SIGN-Lewis-x Complex

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Dendritic Cell-Specific Intracellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) is a C-type lectin highly expressed on the surface of antigen presenting dendritic cells. DC-SIGN mediates interactions between dendritic cells, pathogens and a variety of epithelia, myeloid cells, and endothelia by binding to high mannose residues on pathogenic invaders or fucosylated residues on the membranes of other immune cells. Full length DC-SIGN is a type II membrane protein that consists of a cytoplasmic tail, a transmembrane helix, and an extracellular portion consisting of an α-helical tetramerization stalk plus a C-terminal C-type carbohydrate-recognition domain (CRD). Here we examine the structural characteristics of the interaction of the DC-SIGN CRD with a common fucosylated entity, the Lewis â'trisaccharide (Leâ) using NMR methods. Titration of the monomeric DC-SIGN CRD with Le â monitored by 2D NMR revealed significant perturbations of DC-SIGN crosspeak positions in 1H-N15 heteronuclear single quantum coherence (HSQC) spectra. Additionally, saturation transfer difference (STD) and transferred nuclear Overhauser effect (tNOE) NMR experiments directed at identifying binding epitopes and bound conformations of the Le â ligand were conducted using a tetrameric form of DC-SIGN composed of the CRD plus the α-helical tetramerization domain of DC-SIGN. The constraints derived from these multiple experiments were used to model the binding of Le to the DC-SIGN CRD with HADDOCK. The models were ranked based on fit of the tNOE data and STD buildup curves using the CORCEMA-ST protocol. The best models are presented.

(157) A mucin-binding lectin from skin secretion of Andrias davidianus

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A mucin-binding lectin (ADL) was purified from skin secretion of Andrias davidianus by affinity chromatography on mucin-binding albumin and following gel filtration on Sephadex G-100 and HPLC on TSK-4000. The purified lectin was found to be a dimeric protein, as revealed by SDS-PAGE and MALDI-TOF analysis. SDS-PAGE showed that the ADL protein had a molecular mass of 17 kDa. ADL was found to be composed of 9 kDa band when examined using
(158) Structural and binding properties of the galectins CvGal1 and CvGal2 from the eastern oyster (Crassostrea virginica)

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Galectins are highly conserved lectins that are key to multiple biological functions, including development processes and regulation of innate and adaptive immune responses. Two galectins (CvGal1 and CvGal2) with four tandemly arrayed carbohydrate recognition domains (CRDs), were identified in the eastern oyster (Crassostrea virginica). CvGal1 recognizes a variety of potential microbial pathogens and unicellular algae, preferentially binding to the parasite Perkinsus marinus trophozoites. Further, CvGal1 expressed in phagocytic hemocytes is "hijacked" by the parasite P. marinus to enter the host, where it proliferates, and causes systemic infection and death. By the use of glycan array analysis, ELISA, flow cytometry, SPR, and homology modeling, we compared the protein structure and binding properties of CvGal2 to CvGal1. Like CvGal1, CvGal2 binds to the cell surface of oyster hemocytes and to C. virginica. Glycan array analysis suggested that unlike CvGal1 that recognized preferably blood group A tetrasaccharide present on hemocyte surface, CvGal2 recognized both blood group A and B tetrasaccharide structures. In addition, SPR analysis demonstrated the significant differences in the binding kinetics of CvGal1 and CvGal2 to asialofetuin and a neoglycoprotein displaying the blood group A trisaccharide. The results suggest that CvGal2 has broader binding specificity. CvGal2 is expressed in various tissues of eastern oyster, but is particularly abundant in both circulating and tissue hemocytes. CvGal2 expression is downregulated in parasite-infected oysters. However, parasite binding to hemocytes by addition of exogenous CvGal2 is higher than by CvGal1. Current studies are aimed at assessing potential differences in the binding of CvGal1 and CvGal2 to glycoconjugates on the hemocyte surface, P. marinus trophozoites, and microbial pathogens. (Supported by grants IOS-0822257 and IOS-1063729 from the National Science Foundation, and grant R01GM070589-06 from the National Institutes of Health to GRV, and grant R01 GM080374 from the National Institutes of Health to LXW. We are grateful to Dr. David Smith and Dr. Jamie Molinaro from the Core H, Consortium for Functional Glycomics, Emory University, Atlanta, GA, for the glycan array analysis of CvGal2).

(159) DC-ASGPR isoforms

Gerardo Espino, Sandy Zurawski, Chao Gu, Dorothee Duluc, Sangkon Oh, Gerard Zurawski

Recent genome-wide analyses of alternative splice forms indicate that 40 – 60% of human genes have alternative splice forms. The aim of this work is to study the different alternative splice forms of the human gene CLEC10A (C-type lectin domain family 10, member A). It is reported that this gene is able to produce different splicing variants, four of them be described in literature as: DC-ASGPR (short), DC-ASGPR (long), HML (Human macrophage c-type lectin) and MGL (Human macrophage lectin specific for galactose/N-acetylgalactosamine). It is currently unknown if any stimulus promotes differential expression of any of the splicing variants, or if the isoforms vary in their target recognition. The aim of this work is to study if there are potential divergences in recognition by a panel of in-house anti-DC-ASGPR monoclonal antibodies, ligand binding profile between the alternative splice forms of CLEC10A and some possible differences in effect/biological relevance on other cells that express in their surface the receptor CD45, which is reported to be recognized by the MGL splice isoform. In this report we show a summary of our current research, from the isolation of the different splice variants, to quantitating their relative expression levels, through the design and development of new reagents, and the use of Glycan Array technology to answer some of our primary questions.

(160) Human lung counter-receptors for Siglec-8 and Siglec-9

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Siglecs (sialic-acid-binding immunoglobulin-like lectins) are regulatory molecules expressed on selective subsets of immune cells. Siglec-8 is expressed on allergic inflammatory cells (eosinophils, mast cells and basophils) involved in asthma, whereas Siglec-9 is expressed on neutrophils, monocytes and certain T-cells involved in COPD (chronic obstructive pulmonary disease). Ligation of Siglec-8 on human eosinophils results in their apoptosis, whereas ligation of Siglec-9 results in the same outcome for neutrophils. Presumably, when siglec-expressing inflammatory cells encounter the appropriate polyvalent sialoglycans (counter-receptors) on target tissues, the ongoing response is damped. To search for endogenous Siglec-8 and Siglec-9 counter-receptors in human lung, glycoproteins were extracted from different human lung tissue compartments, from cultured primary human tracheal epithelial cells and gland cells, and from the human lung cell line CaLu-3. Siglec blotting of gel-resolved glycans revealed sialic acid-dependent Siglec-8-Fc binding to a narrow set of high molecular weight sialoglycans only in extracts from human upper airways (trachea and bronchus). The majority of airway-associated Siglec-8 counter-receptor was recovered only by guanidinium chloride extraction, even after detergent extraction. Siglec-8-Fc overlay of human airway tissue sections revealed selective binding to serous cells of the submucosal gland.
Consistent with these findings, human tracheal and bronchial epithelial cells in culture did not express Siglec-8 counter-receptors, whereas human lung subepithelial gland cells and the related human lung cell line Calu-3 did. Preliminary experiments using metabolic inhibitors suggest that the Siglec-8 counter-receptors on cultured cells may be O-linked sialylated glycans. In contrast to Siglec-8 binding, Siglec-9 bound to a number of components of various molecular weights in extracts of human airways, lung parenchyma, and cultured primary human airway epithelial cells and gland cells. Siglec-9 counter-receptors were readily extracted with detergents, and most did not require guanidinium chloride for solubilization. These data further differentiate Siglec-8 and Siglec-9 counter-receptors in the human lung. Characterization of Siglec-8 and Siglec-9 counter-receptors will be important in the study of cell-cell interactions that mediate lung inflammation in health and disease. Supported by the Lung Inflammatory Disease Program of Excellence in Glycoscience (LID-PEG), NHLBI program project grant HL107151.

(161) Characterization of O-GlcNAc modified proteins expressed by E. coli
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O-linked B-N-acetylglucosamine (O-GlcNAc) is a post-translational modification that modifies cytosolic and nuclear enzymes (Wells et al., 2001). As a dynamic and regulatory modification, O-GlcNAc plays a role in numerous pathways and has been implicated in major diseases, including heart disease, cancers, neurodegenerative diseases, and diabetes mellitus (Hanover et al., 2010; Lazarus et al., 2009; Slawson and Hart, 2011; Zachara, 2012). Even though it is conservatively estimated that 30% of the human proteome is regulated by O-GlcNAc (Gupta and Brunak, 2002), only about 2000 proteins have been identified with the modification, about 250 proteins have at least one O-GlcNAc site mapped to a specific residues (Hahne et al., 2013; Wang et al., 2011), and no 3-dimensional (3D) structures of O-GlcNAc-modified proteins have been determined according to the RCSB Protein Data Bank (Kouranov et al., 2006). The goal of our research is to optimize an E. coli expression system for the production of O-GlcNAc modified proteins for NMR. Binding interactions, stability, proteolysis resistance as well as site mapping of the O-GlcNAc modification will also be studied.

(162) Structural insights into the human O-GlcNacase C-terminal domain
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The dynamic modification of proteins by O-linked N-acetylglucosamine (O-GlcNAc) is an essential posttranslational modification present in higher eukaryotes. Removal of O-GlcNAc is catalyzed by O-GlcNAc hydrolase (OGA), a multi-domain enzyme that has been reported to be bi-functional, possessing both glycoside hydrolase and histone acetyltransferase (AT) activity. Insights into the mechanism, protein substrate recognition and inhibition of the hydrolase domain of human OGA (hOGA) have been obtained via the use of the structures of bacterial homologues. However, the molecular basis of AT activity of OGA, which has only been reported in vitro, is not presently understood. Here we describe the crystal structure of a close homologue of the hOGA C-terminal AT domain (hOGA-AT), that we identified in the genome of the marine bacterium Oceanicola granulosus. The structure of this putative acetyltransferase OgpAT in complex with acetyl coenzyme A (AcCoA) reveals that, by homology modeling, hOGA-AT adopts a variant acetyltransferase fold. The structures, together with mutagenesis and SPR data, reveal that while the bacterial OgpAT binds AcCoA, the hOGA-AT does not, as explained by the lack of key residues normally required to bind AcCoA. Thus the C-terminal domain of hOGA is a catalytically incompetent "pseudo" acetyltransferase.

(163) Monoclonal Antibody H is a Novel O-GlcNAc Specific Antibody that Recognizes a Stress-Dependent Epitope on Vimentin
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The modification of nuclear, cytoplasmic, and mitochondrial proteins by O-linked b-N-acetylglucosamine (O-GlcNAc) is an essential post-translational modification of metazoans. O-GlcNAc is thought to regulate proteins in a manner analogous to protein phosphorylation, and like phosphorylation O-GlcNAc levels respond dynamically to various intra-and extracellular stimuli. Recently, we have demonstrated that O-GlcNAc is a novel regulator of the cellular stress response, this conclusion is based on data which demonstrates that: 1) O-GlcNAc levels respond dynamically in response to numerous forms of cellular stress; 2) elevating O-GlcNAc levels promotes cell survival in models of cellular injury, and conversely reducing O-GlcNAc levels sensitizes cells; and 3) numerous pathways within the cell are regulated by O-GlcNAc in a manner consistent with stress tolerance. Notably, O-GlcNAc appears to be protective in models of ischemia reperfusion injury, suggesting that understanding the mechanisms by which O-GlcNAc protects cells will lead to the development of novel therapeutics for treating myocardial infarction and other forms of tissue injury. In order to determine the mechanism by which O-GlcNAc is protective, and how this is mis-regulated in disease, we must identify proteins whose O-GlcNAcylation status changes in response to stress and study the function of O-GlcNAc on these proteins. Such studies will be facilitated by developing and characterizing antibodies that recognize O-GlcNAc in a site-specific manner. Here we demonstrate that the antibody known as monoclonal antibody H, raised against oligodendrocytes, appears to recognize an O-GlcNAc modification site on the intermediate filament protein Vimentin. Moreover, this O-GlcNAc dependent epitope is responsive to numerous forms of cellular injury such as oxidative stress and DNA damage. Current work is focused on identifying the sites of glycosylation and identifying other proteins recognized by this antibody.
Annual Conference of the Society for Glycobiology

(164) The nutrient sensor O-GlcNAc transferase is a critical component of the C. elegans innate immune system

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O-GlcNAc transferase (OGT-1), the enzyme responsible for the addition of the O-linked N-acetylglucosamine (O-GlcNAc) posttranslational modification, has been implicated in both the stress-and immune-response. Furthermore, mammalian OGT-1 physically interacts with evolutionarily conserved proteins key for the immune response (e.g., p38 MAPK a.k.a. PMK-1) and in some cases is known to modify these critical players (e.g., beta-catenin a.k.a. BAR-1). Given these findings and the modification’s role as a signaling molecule, we hypothesized that O-GlcNAc plays a role integrating cues for the innate immune response. Utilizing the bacteriovore C. elegans, we explored whether OGT-1 null animals would stimulate effective immune responses to pathogenic S. aureus (SA). We monitored OGT-1, PMK-1, and BAR-1 mutants under normal conditions and after exposure to SA. All three mutants have decreased viability in comparison to wild type (WT) when fed SA. Importantly, SA-fed PMK-1 null animals in the OGT-1 null background have lifespans that decrease further than the single mutant lifespan suggesting that OGT-1 and PMK-1 act synergistically to stimulate the immune response to pathogenic SA. Interestingly, BAR-1 null animals in the OGT-1 null background have lifespans that are similar to the single mutants suggesting that these immune system components are in the same pathway. In addition, we generated genome-wide microarray data to analyze the OGT-1 and PMK-1 null animals’ transcriptional response to SA. These data indicate that there is little overlap between the genes regulated by OGT-1 and PMK-1 supporting that they act in different pathways to promote immunity. Indeed, both OGT-1 and PMK-1 control the expression of candidate antimicrobials including C-type lectins, antimicrobial peptides, and CUB-like genes. Our data highlight O-GlcNAc as a novel, indispensable player in the C. elegans innate immune response to SA and that OGT-1 is an essential mediator of the expression of SA-triggered immune response genes. Our findings underscore the need to bolster our understanding of immune-response signaling pathways in order to combat pathogen infection.

(165) The Enzymes of the O-GlcNAc Cycling: Writers AND Readers of the Histone Code?

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NIH/ NIDDK

Epigenetic regulation of gene expression is essential for a multitude of processes in an organism and aberrant transcription can result in disease development. Better understanding of how transcriptional profiles are regulated, particularly in response to external signals such as nutrient supply, could aid in development of methods to better detect, treat, or prevent disease. One way cells regulate gene expression is through posttranslational modification (PM) of histones. An often-overlooked PM is the O-GlcNAcylation of serine/threonine residues. O-GlcNAc is added and removed by a single pair of enzymes, O-GlcNAc transferase (OGT) and O-GlcNAc case (OGA), respectively. O-GlcNAc addition is the final step of the nutrient driven hexosamine-signaling pathway, and as such it is likely that O-GlcNAc addition/removal informs upon an organism’s nutritive state. Interplay between O-GlcNAcylation and other PMs on histones may allow cells to fine-tune transcriptional activity in response to cellular cues. To examine this possibility, we are focusing on the interaction of OGA and OGT with both known modifiers of histone PMs and modified histones themselves. Previous studies suggest that OGT plays a role in down regulating gene expression by binding and O-GlcNAcylation the SIN-3 histone deacetylase complex. We recently demonstrated through transcriptional analysis in Caenorhabditis elegans that about half of the genes that are deregulated when OGA or OGT are absent are also deregulated when SIN-3 is absent. We are taking a genetic approach using these knockout Caenorhabditis elegans strains to further characterize the relationship between the enzymes of O-GlcNAc cycling, SIN-3 and histone acetylation. These studies will provide insight into how the nutritive state is translated into changes in transcriptional profiles on the level of an entire organism. In addition, OGA is known to possess a putative histone acetyltransferase (HAT) domain, prompting us to question what role it might play in OGA/ histone interactions. Using recombinant OGA protein and differentially modified histone tail peptides, we have identified specific modifications with well known roles in transcriptional regulation to which OGA exhibits binding selectivity. Further characterization of these binding interactions will allow for the us to begin "decoding" the intricate relationship between PMs, transcriptional regulation, and intermediary metabolism.

(166) Defining the mechanism of O-GlcNAc signaling in stress-induced autophagy and cardioprotection

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Ischemic heart disease causes significant morbidity and mortality worldwide. Therapies for treating myocardial infarction and ischemia reperfusion injury (IR/ I) have thus far remained elusive. Current research demonstrates that the dynamic modification of intracellular proteins by O-linked -N-acetylglucosamine (O-GlcNAc) confers cardioprotection in models of IR/ I. However the molecular mechanism(s) by which O-GlcNAc promotes survival of the myocardium have not been elucidated. In this study we have investigated the hypothesis that O-GlcNAc regulates the cardioprotective process of autophagy. As many techniques used to modulate protein expression (RNAi, viral transduction) induce autophagy, we have generated a series of tools that facilitate conditional control of cellular O-GlcNAcylation. Utilizing destabilizing domain technology (ProteoTuner, Clonetech), we have generated chemically inducible constructs that regulate the protein levels of the enzymes that catalyze the addition and removal of O-GlcNAc, the O-GlcNAc transferase (OGT) and the O-GlcNAcase. These constructs and their catalytically dead counterparts have been stably transfected into cells and characterized. We have shown that enhancing global O-GlcNAcylaton increases autophagic flux, whereas reducing O-GlcNAc levels, via O-GlcNAcase overexpression, suppresses the induction of autophagy in a model of
oxidative stress. Consistent with these observations, we have demonstrated that AMP kinase signaling, which promotes autophagy, is upregulated when O-GlcNAc levels are increased pharmacologically.

To tease out the mechanisms by which O-GlcNAc regulates autophagy we have assessed the O-GlcNAc modification state of key regulators of autophagy. AMPK, ULK1 and p62/sequestosome are O-GlcNAc modified or associate with O-GlcNAc modified proteins. Importantly, AMPK appears to be directly O-GlcNAc modified in a stress-dependent manner and associates with OGT. These data suggest that O-GlcNAc may regulate autophagy by modifying and regulating AMPK directly. Together, our data suggests that O-GlcNAc regulates numerous points in the autophagic pathway and may promote cardioprotection through the upregulation of autophagy.

(167) Elucidating the role of O-GlcNAcylation on RUNX2-mediated transcriptional programs in bone-marrow derived mesenchymal stem cells

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The runt-related transcription factor 2 (RUNX2) activates osteogenic transcriptional processes key to bone development, and its function is modulated by post-translational modifications such as phosphorylation and acetylation. A single study [Kim et al. (2007) Biochemical and Biophysical Research Communications, vol. 362, pp. 325-329] suggests that RUNX2 is modified by O-linked N-acetylglucosamine, a post-translational mechanism which modulates nutrient-and stress-induced transcriptional pathways. Supporting the hypothesis that O-GlcNAc-mediated signaling contributes to bone formation, we have previously identified a number of O-GlcNAcylated proteins in differentiating osteoblasts. Here we confirm the O-GlcNAc modification of exogenous RUNX2, and employ a click-chemistry based enzymatic labeling strategy to demonstrate multiple sites of O-GlcNAc modification in osteogenic bone marrow mesenchymal stem cells (BMMSCs). Further supporting a role for O-GlcNAc in the regulation of osteogenesis, we observe significantly enhanced activity of the matrix maturation marker alkaline phosphatase (ALP) in osteogenic BMMSCs cultured in the presence of Thimet G, a selective inhibitor of the O-GlcNAcase (OGA) cycling enzyme. These data suggest that inhibiting OGA activity enhances osteogenesis in BMMSCs. Continuing studies are focused on determining the upstream factors which regulate OGA/ O-GlcNAc transferase activity and RUNX2 O-GlcNAcylation during osteogenesis, as well as elucidating exact sites of RUNX2 Ser/ Thr O-GlcNAcylation by tandem mass spectrometry. This work is supported by NIH/NIDCR grant nos. RO1-DE020925-01A1 and T32-DE017551-05.

(168) Protein O-GlcNAcylation Regulates β-catenin, E-cadherin and Cell Motility

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The Wnt/β-catenin signaling pathway and cadherin-mediated adhesion are implicated in epithelial-mesenchymal transition (EMT), a key cellular process in invasion and metastasis. Often, deregulation of the Wnt pathway is caused by altered phosphorylation of its components. Specifically, phosphorylation of Ser or Thr residues of β-catenin affects its location and interaction with E-cadherin, thus facilitates cell-cell adhesion. O-GlcNAcylation, the addition of O-GlcNAc to Ser or Thr, is comparable with phosphorylation and may compete with it on the same or adjacent residues. Consistently with previous studies, the current study indicates that β-catenin and E-cadherin are O-GlcNAcylated. To test the effect of O-GlcNAcylation on cell motility and how O-GlcNAcylation might affect β-catenin and E-cadherin functions, O-GlcNAcase, the enzyme responsible for the removal of O-GlcNAc, was inhibited. This inhibition resulted in a global elevation of protein O-GlcNAc modification, increased expression of E-cadherin and β-catenin, and altered β-catenin translocation in fibroblasts. Concomitantly with O-GlcNAcylation elevation, fibroblast cell motility was enhanced. The effect of O-GlcNAcylation on β-catenin transcriptional activity is now under investigation. The results described herein may support the notion that O-GlcNAcylated β-catenin and E-cadherin, which may further influence metastasis and wound healing.

(169) OGT is an X-linked imprinted gene linked to Cardiovascular Disease Susceptibility

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Turner Syndrome (or monosomy X, X,45) occurs in 1 of 2500 live female births and is the most common genetic disorder effecting women. Some Turner patients display a complete loss of either the paternally - or maternally-derived X chromosome. Women monosomic for a maternally derived X (Xm) have android patterns of fat deposition, atherogenic metabolic profile and increased susceptibility to heart disease compared to women monosomic for a paternally transmitted X (Xp). Our hypothesis is that the parent-of-origin of the monosomic X in Turner Syndrome patients may be diagnostic of their risk for developing metabolic and cardiovascular disease. We have identified changes in gene expression associated with inheritance of Xp or Xm and have validated a number of biomarkers including the genes XIST and OGT as candidate genes which influence metabolic programming. OGT is of interest because it has been implicated in nutrient-dependent chromosome remodeling and genomic imprinting. Recently, OGT has been shown to interact with the Tet family of enzymes that catalyse the conversion of 5-methylcytosine of DNA to 5-hydroxyl-methylcytosine. We are pursuing this observation using iPS technology and mouse genetics. The present findings will have importance for the diagnosis and identification of Turner syndrome patients at high risk for the development of metabolic and cardiovascular disease. Identification of OGT as an X-imprinted gene may also have profound therapeutic implications. Our findings enable a dissection of the role of the human
X-chromosome in metabolic and cardiovascular disease in the absence of confounding sex hormonal influences and identified OGT as an X-imprinted therapeutic target.

(170) Role of O-GlcNAc in leukemogenic nucleoporin fusion activity
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Onset of leukemia is associated with chromosomal translocations. Notably, 28 known chromosomal translocations involve nucleoporin 98 (NUP98), and result in fusion proteins encoding the N-terminus of NUP98 fused to homeodomain transcription factors or other proteins that interact with nucleic acids. The mechanism of how these fusion proteins transform cells to a cancerous state is not well understood. Nucleoporin proteins (NUPs) are components of the nuclear pore complex that facilitate nuclear trafficking. Disruption of this process can lead to altered cellular homeostasis and disease pathogenesis. At least one-third of NUPs contain phenylalanine-glycine (FG) repeat domains, which are natively unfolded and highly modified by O-linked N-acetyl-D-glucosamine (O-GlcNAc). This modification on the FG repeats may regulate the function and binding interactions of NUP98. Furthermore, the NUP98 fusion proteins exhibit altered localization, likely affecting nuclear trafficking and providing a potential mechanism for disease onset. NUP98-HOXA9 and NUP98-DDX10 are among the NUP98 chromosomal translocations discovered in leukemia patients. Therefore, I am characterizing the binding interactions facilitated by the FG repeat domains in NUP98 when fused to HOXA9 and DDX10. These proteins are difficult to study with traditional techniques as they involve highly glycosylated, inherently disordered protein domains. However, I am utilizing a novel chemical tool developed in the Kohler lab to overcome this difficult biological problem. This method allows me to introduce a photocrosslinking functional group (diazirine) on the O-GlcNAc modification in living cells, using the chemically modified sugar, GlcNDAz. Unlike traditional crosslinking, GlcNDAz provides specific and covalent crosslinks to identify binding partners mediated by O-GlcNAc. In preliminary work, I synthesized the photocrosslinking sugar precursor (GlcNDAz-1-P) on multi-gram scale. My initial crosslinking data show that NUP98 fusions proteins successfully crosslink to unknown target(s) in cell lines derived from both leukemia and cervical carcinoma. Further studies will be conducted to identify target(s) and determine the interactions’ functional roles during leukemogenesis.

(171) Defining binding affinities and specificities for anti-blood group A antibody
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Anti-blood group antibodies are present in people lacking the corresponding antigens; thus the immune response from a mismatched transfusion can lead to hyperacute immune response and death. Blood group A and B trisaccharide antigens differ only in the substitution of an acetamido group for a hydroxyl group. The ability of antibodies to differentiate between these antigens therefore depends on a remarkable level of specificity and affinity for a relatively modest structural difference. Experimental methods like X-ray crystallography and NMR can be used for characterizing these complexes. However, these methods are laborious, costly, and the degree of success is unpredictable. In this study, we employed a combination of molecular docking (VINA-Carb), molecular dynamic simulation (MD), and energy calculations (MM-GBSA) to understand the affinities and specificities of an antibody scFv fragment (PDB ID: 1JV5) specific for blood group A antigen, as well as to understand the origin of it’s lack of affinity for B[1]. The combination of these methods can be a very powerful tool for prediction of antibody-carbohydrate complexes, which are otherwise difficult to characterize.

(172) Toward a new class of immunosuppressive drugs: Identification and characterization of ST6Gal1 sialyltransferase inhibitors
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The α2-6 sialyltransferase ST6Gal1 elaborates ligands for the B cell siglec CD22, which is involved in the regulation of B cell receptor signaling. B cells of ST6Gal1-deficient mice exhibit a hypo-immune response, which suggests that this enzyme may be a promising target for the development of inhibitors intended to suppress immune responses and treat autoimmune disease. Based on a large-scale screen for inhibitors of two sialyltransferases, we have conducted a systematic analysis of active compounds to identify potent and selective inhibitors of ST6Gal1 as potential leads for drug development. Approximately 364,000 compounds were

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**Fig. 1.** Blood group antigen A and B differ only by a single substitution. Antigen A (GalNAcα1-3(Fucα1-2)Galβ) is shown on the left and antigen B (Galα1-3(Fucα1-2)Galβ) on the right.
screened for activity against human ST6Gal1 through the Molecular Libraries Probe Production Centers Network (MLPCN) at the Scripps Research Institute, Florida. More than 900 hits were identified based on greater than 50% inhibition at 6 uM in vitro. The hits were analyzed in silico based on shared structural motifs, highlighting 325 compounds that had high potency, selectivity for ST6Gal1 over ST3Gal1, and the potential for optimization by medicinal chemistry. Of these, 170 were further validated in vitro using a high-throughput (HTP) fluorescence polarization assay to construct dose-response profiles against ST6Gal1. Selectivity was also confirmed by comparison against ST3Gal1, a related sialyltransferase family member. An orthogonal radioactivity-based assay was then used to validate the most potent and selective inhibitors from the HTP screen. Results highlighted 25 inhibitors with potency in the low uM range and high selectivity for ST6Gal1 in vitro. The results from this in vitro profiling study lay the foundation for a detailed structure-function analysis that may support the rational design of small molecule therapeutics targeting ST6Gal1. Funded by Janssen Pharmaceuticals (SFP-2064) and the NIH (A1050143, MH-084512).

(173) Sialic acids in marine bacteria

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By analyzing the genomes of the two marine fish pathogenic bacteria Aliivibrio salmonicida LFI1238 and Moritella viscosa 06/09/139 we discovered that they contain the complete genetic machinery to synthesize N-acetyl neuraminic acid. The presence of this sialic acid is in other bacteria very important for survival in the host. The sugar is thought to act as a cloaking device, masking the bacteria from the host’s immune system as the terminal glycans on vertebrate cell surfaces are dominated by sialic acid. Recently we have published data that suggests a novel pathway for bacterial synthesis of 7-O-acetylated sialic acids (Gurung et al. 2013. Glycobiology. 23: 806-819). Currently, using HPLC-MS and bioinformatics, we are analyzing the modifications (acetylation) of sialic acid done by the bacteria and try to pinpoint the genes involved. Through recombinant protein production and x-ray crystallography we are characterizing the enzymes leading to sialic acid from these bacteria. Enzyme pathways leading to two related sugars called legionaminic acid and pseudaminic acid are also under investigation. In vitro production of various sialic acid analogues is in addition being pursued.

(174) Flagellin/Toll-like receptor 5 response was specifically attenuated by keratan sulfate disaccharide via decreased EGFR phosphorylation in normal human bronchial epithelial cells

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COPD, which is predicted to be the third leading cause of death by 2020, is associated with specific inflammatory responses around small airways. COPD exacerbation occurs in some patients mostly due to bacterial or viral infections of the lung and air pollution. In fact, highly significant levels of bacteria are observed in the airway of exacerbated COPD as compared to those in healthy adults. Bacterial infection is associated with the pathogenesis and exacerbation of COPD partly via the induction of host inflammatory responses. Thus, it can be a potent therapeutic target for COPD to suppress airway inflammatory responses triggered by bacterial infection. Keratan sulfate (KS) is the major glycosaminoglycans of airway secretions, and is synthesized by epithelial cells on the airway surface. The findings reported herein show that a KS disaccharide, [ SO3-6]Gal9-4[SO3-6]GlcNAc, designated as L4, suppressed the production of IL-8 stimulated by flagellin, a Toll-like receptor agonist, in normal human bronchial epithelial (NHBE) cells (Shirato et al. 2013). Such suppressions were not observed by other L4 analogues, N-acetyllactosamine or chondroitin-6-sulfate disaccharide. Moreover, treatment of NHBE cells with L4 inhibited the flagellin-stimulated phosphorylation of epidermal growth factor receptor, the down stream signaling pathway of TLRs in NHBE cells. These results suggest that L4 specifically blocks the interaction of flagellin with TLR5 and subsequently suppresses IL-8 production in NHBE cells. Taken together, L4 represents a potential molecule for prevention and treatment of airway inflammatory responses to bacteria infections, which play a critical role in exacerbation of COPD.

Reference


(175) Mechanisms of pneumococcal neuraminidases in facilitating host infection

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Despite the introduction of Streptococcus pneumoniae (pneumococcus) vaccine thirteen years ago, pneumococcus remains a highly infectious pathogen, and the main cause of bacterial meningitis in children and older adults. In addition, pneumococcus is increasingly resistant to antibiotics, highlighting the need for more effective therapeutics. Thus, a better understanding of pathways pneumococcus exploits in initiating host infection is vital to improving available treatments and diminishing pneumococcal meningitis-related deaths. Importantly, pneumococcus utilizes a number of glycosidases in its genome to modify host glycosylation and promote infection. Of these, three are neuraminidases, which cleave sialic acid from extensive glycan chains decorating cell surfaces. This function has the potential to reveal sites for host-pathogen interactions, enable evasion of the immune system, or alter inter- and intracellular signaling pathways to facilitate progression of pathogenesis. While pneumococcal neuraminidases have indeed been shown to facilitate adherence, invasion, and blood-brain barrier crossing, the mechanism of their contribution to infection remains unknown. I hypothesize that pneumococcal neuraminidases unmask glycoproteins on
the blood-brain barrier that are necessary for invasion and transcytosis by pneumococcus. In order to identify proteins in the blood-brain barrier that are de-sialylated by pneumococcal neuraminidases, I have utilized a recently developed glycoproteomics technique on a blood-brain barrier model cell line, hCMEC/ D3. In brief, galactose residues exposed upon neuraminidase treatment of hCMEC/ D3 cells are oxidized to introduce aldehyde groups, which are then labeled with aminooxy biotin using an aniline-catalyzed ligation. Tagged proteins are subsequently streptavidin purified and subjected to mass spectrometry for identification. Using this technique, I have detected enrichment of ~60 proteins in neuraminidase-treated hCMEC/ D3 cells over that of untreated cells. Currently, I am assessing the functional significance of these substrates on pneumococcal infection. To do this, identified protein targets will be knocked down in hCMEC/ D3 cells using shRNA. Next, I will determine the effect of each protein on pneumococcus adherence, invasion, and transcytosis of the blood-brain barrier. Importantly, this work has the potential to reveal new targets for therapeutic development as well as provide unique insights into the basic biology of bacterial meningitis.

(176) Role of soluble heparan sulfate proteoglycans in sepsis
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Sepsis is the most common cause of death among hospitalized patients in non-cardiac intensive care units. In severe sepsis, systemic inflammation often leads to hyper-activation of the coagulation system, microvascular thrombosis and organ dysfunction, causing a life-threatening syndrome known as disseminated intravascular coagulation (DIC). Heparan sulfate proteoglycans (HSPGs) expressed on the blood-vessel wall interact with a wide range of inflammation-related factors, such as chemokines and proteases. Given the clinical use of heparin, a soluble analog of heparan sulfate (HS), as an anti-coagulant, we hypothesized that HSPGs could play an important role in coagulation system during sepsis. To understand this phenomenon further, we took advantage of the mouse model conditionally-deficient in EXT1 gene function by inducible and Tie-2 (myeloid/ endothelial cell) specific-Cre/ loxP recombination systems (Immunity; 33: 817-29, 2010). In analyses of hemostasis, mice with cell-type specific loss of EXT1 function showed a shortened prothrombin time (PT) and activated partial thromboplastin (aPTT) time, indicating that HS normally functions as an endogenous anti-coagulant. When wild-type mice were challenged intraperitoneally with the Gram-positive bacterial pathogen Streptococcus pneumoniae (SPN, strain D39) or orally with Gram-negative Salmonella enterica Typhimurium (ST), the level of syndecan-1/4 shedding into plasma was significantly increased. It is known that bacterial metalloproteinase (MMP) sheds HSPGs. When mice were treated with MMP inhibitor GM6001 following bacterial infection, HSPG shedding in plasma of septic mice was significantly inhibited. Interestingly, mice treated with GM6001 after ST infection showed no detectable bacteria in the blood and no disease manifestations at a late stage infection time point, whereas mice that did not receive the MMP inhibitor had high levels of bacteria in the blood and severe disease manifestations, with mortality following shortly after. In contrast, GM6001 worsened the progression of SPN-mediated sepsis. While the EXT1-KO mice showed prolonged survival than WT mice in SPN-sepsis, those mice showed shortened survival than WT mice when challenged with ST. These results suggest that soluble form of HSPGs have distinct roles that are dependent on bacterial species causing sepsis. Together findings suggest that HS is involved in the coagulopathy and disease progress during sepsis. Supported in part by NCI PO1CA71932 grant.

(177) Proto-type galectins from teleost fish are secreted into skin and gut mucus, and bind to bacteria
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Like all aquatic vertebrates and invertebrates, teleost fish are subject to the constant pressure of bacterial, fungal and parasitic organisms present in the environmental interface, and that can potentially cause disease. Numerous defense molecules have been isolated from the skin and gut mucus of various fish species. To provide new insights into the potential role(s) of galectins in the teleost fish innate immune system, we selected the striped bass (Morone saxatilis), a teleost fish species of environmental and economic relevance in the Chesapeake Bay, and amenable to biochemical, molecular, and histological approaches, particularly concerning skin and intestinal mucus. We identified in skin mucus, and subsequently purified from skin and muscle tissue a 15 kDa galectin-like protein which we designated MsGal15. Analyses of its primary structure and carbohydrate specificity indicate that MsGal15 is closely related to the mammalian galectin-1. The exon-intron boundary in MsGal15 gene is conserved when compared with the mammalian galectin-1 and other teleost proto type galectins from vertebrates. MsGal15 directly binds to various bacterial species and strains, such as Vibrio anguillarum and V. mimicus, which are potential pathogens of striped bass. Histological examination indicated that MsGal15 is expressed in connective tissue, resident macrophages, circulatory leukocytes, and rodlet cells. Electron microscopy with immunogold reveals numerous positive signals in the cytoplasm of cells that morphologically resemble intestinal macrophages, as well as clustering of MsGal15 on the surface of intestinal microvilli. With the goal of investigating the biological role(s) of the aforementioned galectins in a genetically tractable system, and using the molecular biochemical and serological tools developed with M. saxatilis, we examined...
the presence of similar galectins in skin and mucus of the zebrafish (*Danio rerio*). Western blots of zebrafish skin extracts and mucus developed with anti-MsGal15 antibodies revealed a component of electrophoretic mobility corresponding to 15 kDa, similar to that observed for MsGal15 in skin and mucus. Current efforts are directed towards cloning the putative zebrafish MsGal15 equivalent, and using genetic approaches to rigorously examine its biological role in defense functions against infectious challenge (Supported by grant 5R01GM070589-06 from the National Institutes of Health to GRV).

(178) Exploring the sialoglycan binding properties of a family of bacterial adhesins containing a novel Siglec-like domain that contributes to bacterial virulence

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The serine-rich repeat (SRR) glycoproteins of Gram-positive bacteria are a diverse family of adhesins. These molecules play important roles in determining virulence of the bacteria for human infections such as infectious endocarditis (IE). Among the SRR type of bacterial adhesins, GspB of *Streptococcus gordonii* has been found to be a lectin and contain a novel Siglec-like (Siglecl-L) domain in its binding region (BR). Hsa of *Streptococcus gordonii* and SrpA of *Streptococcus sanguinis* are two GspB homologs and the only other known glycan-binding SRR proteins characterized in any detail to date. Interestingly, GspB and Hsa were found to mediate streptococcal binding to human platelets and to be involved in the pathogenesis of IE, but SrpA, in contrast, did not contribute to virulence in an animal IE model. In the present study, we aim to answer three questions: 1) Is there a traceable link between the phylogeny of the bacterial Siglec-L domains and their sialic acid (Sia) binding properties as well as the microbial virulence? 2) Since these bacteria are mainly oral streptococci, what are the relationships between the Sia-adhesin interactions involved in oral colonization and those that contribute to the pathogenesis of IE? 3) How is it possible for these bacteria to bind their cognate Sia ligands on human platelets, in the face of numerous naturally-occurring potential binding competitors in the blood? We will present data towards addressing each of the questions using wild-type and mutant forms of several SRRs as well as corresponding isogenic bacterial strains, applied to a variety of complementary techniques, including sialoglycan microarray, enzyme-linked immunosorbent assay (ELISA), dot blot assay, platelet adhesion assay, hemagglutination assay, and flow cytometry. These studies provide novel insights into the molecular basis for the sialoglycan binding by this novel subgroup of SRR proteins. The results have consequences for the understanding of human infectious diseases involving these adhesins, and might open up novel therapeutic approaches to combat them.

(179) Identification of new binding partners for cholera toxin from *Vibrio cholerae* and VacA from *Helicobacter pylori*

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Exotoxins that are secreted from bacteria cause damage to host cells by destroying cells or disrupting normal cell metabolism. These exotoxins bind host cells and transduce signals, but how they recognize host cells is not well understood. To isolate the relevant low affinity glycan-protein interactions, we developed a photocrosslinking strategy that relies on the metabolic incorporation of diazirine-modified sialic acid residues (SiaDaz) to covalently capture-detected complexes formed between an exotoxin and its sialylated binding partners. In this study, we focused on the identification of novel receptors for cholera toxin from *Vibrio cholerae* and VacA from *Helicobacter pylori*. Although the ganglioside GM1 is widely accepted to be cholera toxin’s sole receptor, there have been increasing reports over the last 40 years suggesting that additional cell-surface binding partners for cholera toxin may exist. Similarly, the host cell receptor for VacA is not well established, but it is suggested that sialic acid is involved. We showed that photocrosslinking of cholera toxin or VacA to SiaDaz-producing intestinal epithelial cell lines followed by immunoblotting results in the appearance of a new toxin-containing higher molecular weight species. To determine the class of glycoconjugate contained in the crosslinked complex, T84 cells or Caco-2 cells were cultured with N-butyldenoxy-galacto-nojirimycin (NB-DGJ) that interferes with addition of glucose to ceramide, if there is which interferes with N-linked glycan biosynthesis, or α-benzyl-GalNAc, a decays substrate that interferes with O-linked glycan production. For both cholera toxin and VacA, we observed depletion of the high molecular weight band in α-benzyl-GalNAc treated cells. We conclude that both cholera toxin and VacA bind O-linked glycoproteins on intestinal epithelial cells. Furthermore, mass spectrometry analysis of the cholera toxin crosslinked complex identified CD44, NKCC1 and integrin β4 as potential cholera toxin binding partners. Next, in order to determine whether the O-linked glycoprotein functions in host cell entry and intoxication, we performed functional assays. T84 cells cultured in the presence of α-benzyl-GalNAc exhibit a decrease of the intracellular cdc25 on monophosphate (cAMP) accumulation and the electrogenic chloride secretion response to cholera toxin compared to control cells. Thus, O-glycans on glycoproteins are important receptors of cholera toxin and influence the following intoxication.

(180) Overlapping and distinct roles of *Aspergillus fumigatus* UDP-glucose 4-epimerases in galactose metabolism and the synthesis of galactose-containing exopolysaccharides

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Aspergillus fumigatus, an ubiquitous filamentous mold, is the main causative agent of invasive aspergillosis in immunosuppressed patients undergoing transplantation or chemotherapy. Although the fungal cell wall is emerging as an effective target for antifungals, our understanding of this complicated structure is still evolving. The cell wall and biofilm matrix produced by A. fumigatus is composed of various polysaccharides, including two galactose-containing exopolysaccharides, galactomannan and the recently characterized galactosaminogalactan. Galactosaminogalactan mediates adherence, masks β-glucans, induces apoptosis and produces immunomodulatory effects, however very little is known about its biosynthesis. We previously found that deletion of an UDP-glucose 4-epimerase, uge3, blocks galactosaminogalactan synthesis, underscoring the importance of this class of enzymes. In A. fumigatus, there are three putative UDP-glucose 4-epimerases. We undertook the present study to elucidate the role of these three UDP-glucose 4-epimerases in A. fumigatus galactose metabolism and the biosynthesis of galactose containing cell wall glycans. In this study we report that uge4 was minimally expressed under all conditions tested and does not seem to contribute significantly to synthesis of galactose-containing exopolysaccharides or galactose metabolism. Uge5 is the dominant UDP-glucose 4-epimerase in A. fumigatus and essential for normal growth in galactose-based media. uge5 is also required for the synthesis of the galactofuranose (Galf) component of galactomannan and a significant fraction of the galactose component of galactosaminogalactan. Uge3 has specificity to both UDP-galactose and UDP-GalNAc, and in the absence of Uge5, Uge3 activity is sufficient for the synthesis of galactosaminogalactan containing reduced amounts of galactose. However, Uge3 activity is not sufficient to support Galf synthesis and the production of galactomannan. A double deletion of uge5 and uge3 blocked growth on galactose and abrogated synthesis of both Galf and galactosaminogalactan. This study is the first survey of glucose epimerases in A. fumigatus, and contributes to our understanding of the role of these enzymes in metabolism and cell wall synthesis.

A predictive model of polysaccharide capsule regulation in Cryptococcus neoformans

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Cryptococcus neoformans is a pathogenic fungus that kills over 600,000 people a year, mainly immunocompromised individuals in developing areas of the world. This yeast bears a protective polysaccharide capsule that is required for its virulence; capsule polysaccharides are also shed from the cell and impair the host immune response to infection. Although the structures of the two main capsule polysaccharides have been determined (Cherniak et al. 1998; Vaishnav et al. 1998; Heiss et al. 2009, 2013), to date only one glycosyltransferase of the many required for their synthesis has been conclusively identified. Capsule thickness varies with environmental conditions, becoming significantly greater during infection. We are reconstructing the transcriptional network that regulates capsule synthesis (Haynes et al. 2011), with the dual goals of understanding how environmental signals are integrated to determine capsule size and defining the downstream machinery responsible for its synthesis. We have used gene expression data from transcription factor (TF) mutants to identify interactions between TFs and their target genes that are relevant to capsule formation. To do this we applied a computational method called NetProphet, which we have shown can reconstruct direct transcriptional regulation networks with accuracy comparable to that of direct in vivo binding methods like chromatin immunoprecipitation (ChIP) (Haynes et al. 2013). We used networks generated by NetProphet to identify candidate TFs that we predicted to impact capsule regulation; deletion of these factors has validated many of our predictions. We have additionally used the networks to identify genes encoding proteins that we expect to act directly in capsule synthesis. Deletion of these genes has yielded multiple new capsule mutants. We are continuing to probe capsule regulation as well as testing the virulence of our new mutants in animal models of infection and investigating the biochemical roles of the affected gene products.

References


(181) Binding to TLR glycans is involved in the immunomodulatory effect of paracoccin on Paracoccidioides brasiliensis infection

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Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis, the most frequent systemic mycosis in Latin America. We have identified a component in yeast surface of this fungus named paracoccin, which is an N-acetylgalcosamine binding protein that promotes fungal adhesion to extracellular matrix components and induces high and persistent levels TNF-alpha and of nitric oxide production by macrophages. Paracoccin treated mice showed, 30 days after infected with P. brasiliensis, higher levels of Th1 and anti-inflammatory cytokines, in comparison to control mice. Furthermore, microscopy examination of lung sections showed that the paracoccin treatment preserve organ architecture, with scarce compact and well-organized
granulomas, containing few silver-stained yeasts. On the basis of these previous observations, this study aimed to investigate which host molecules are targeted by paracoccin to generate the reported responses. Using a reporter gene system in HEK cells, we have transfected Toll-like receptors into them. TLR1, TLR2, TLR4 and TLR6 transfected cells produced IL-8 when stimulated with paracoccin. The accessory molecules CD14 and CD36 seem to be unnecessary for the activation process. We presently investigate which TLR glycans are recognized by paracoccin and which one(s) is implicated in signaling pathways triggered by paracoccin. Taken together, our results indicate that TLR N-glycans are possibly targeted by paracoccin and account for cell activation and production of the Th1 cytokines that modulate immunity toward a protective pattern against P. brasiliensis infection.

(183) A ricin-resistant mutant of Leishmania donovani with unique lipophosphoglycan composition

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Leishmania parasites manipulate essential interactions with its sand fly vector and vertebrate host by expressing developmentally-regulated forms of its surface lipophosphoglycan (LPG). LPG is a GPI-anchored polysaccharide consisting of repeating 6Galβ1,4Manα1-PO4 units. The number of repeat units varies and their expression is critical depending on the stage of the life cycle: n ~ 15 in procyclic and n ~ 30 in metacytic promastigotes; and n = 0 in the intracellular amastigote forms. Our work focuses on the family of mannosyl-phosphate transferases involved in LPG formation. At least two mannosylphosphoryltransferases (MPTs) have been implicated in repeat unit assembly: an "initiation" specific MPT (iMPT) which uses the galactose (α1,6) of the LPG core as the acceptor and an "elongation" specific MPT (eMPT) which uses the galactose (β1,4) of the repeat units as acceptor. We previously identified a ricin-resistant mutant of L. donovani Sudan that surprisingly expresses the longer "metacytic" length LPG in the procyclic form. Early structural evidence suggests not only is the LPG longer, but includes a glucose-containing cap structure that is uncharacteristic of the wild-type L. donovani and could account for the resistance of the mutant to agglutination by ricin. Enzymatic analyses are being used to further explore these differences in the context of the MPTs.

(184) Biochemical nature of lectin-binding ceramide-phosphatidylinositol glycolipid membrane anchor from Trichomonas vaginalis

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The most common non-viral sexually transmitted infection is caused by the protozoan parasite, Trichomonas vaginalis (TV). The rate of infection is higher than Chlamydia and gonorrhea combined. TV causes major social, economic, and health care burdens due to recurrent vaginitis, preterm delivery, low birth weight, and higher risk of HIV, HPV and cancer. We have shown that the major surface lipophosphoglycan (LPG) and its ceramide-phosphatidylinositol glycan core (CPI-GC) mediate host inflammatory responses to TV. Therefore, defining the biochemical nature of CPI-GC is needed for understanding and controlling the infection and its complications. We have determined that CPI-GCs from several TV-isolates vary in monosaccharide compositions and, as determined by Biolayer Interferometry, vary in their binding to lectins, implying various amounts of LacNAc repeats and/ or degree and substitutions in glycan branching. We have focused our attention on two biologically well characterized strains: UR-1, which shows relatively low binding to galectin-1 and galectin-3 and B7RC2, which shows high binding to both galectins. We also analyzed two mutants derived from B7RC2 (M-412 and M2E2), which show no binding to gal-1 and gal-3. We used MS and NMR, including enzyme and chemical treatments, to define the nature of CPI-GCs. UR-1 CPI-GC showed predominant chains of -3-α-Gal-1-3β-GlcNAc-1-disaccharide repeat units. The presence of terminal GalNAc and terminal α-and β-Gal suggests that the chains are either terminating at the non-reducing end with GalNAc and α-or β-Gal or that the side chains have some GalNAc and α-or β-Gal side chains. The other novel features of this CPI-GC are the presence of phosphoethanol-amine, phospho-choline and phospho-ceramide which were determined by HF treatment followed by MS and NMR analyses of CPI-GC. MALDI-TOF MS analyses of CPI-GCs from B7RC2, M-412, and M-2E2 showed molecular ions centered at m/z ~8160, ~6384, and ~6381 respectively. The mass spectra of the wild type (B7RC2) CPI-GC showed a series of lactosamine fragments whereas the mutants showed no lactosamine, consistent with the occurrence of gal-1 and gal-3 binding. These structural features were accompanied by altered immune-inflammatory responses to the purified CPI-GC glycolipid. (Supported by 2RC1 AI086788, 2RS6 AI091889, 5R01 AI079085, P41 GM104603, S10 ODO10724, DE-FGO-2-93ER-20097).

(185) Microarray Analysis of the Response of Human Antibodies to Synthetic Cryptosporidium Glycopeptides

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Glycoproteins expressed by the protozoan parasite Cryptosporidium parvum, which causes gastrointestinal illness, are immunogenic in infected individuals, but the nature of the epitopes recognized in C. parvum glycoproteins is poorly understood. A known immunodominant antigen of Cryptosporidium, the 17-kDa glycoprotein, has previously been shown to bind to lectins that recognize the Tn antigen (GalNAca1-Ser/Thr-R). Therefore, we prepared a large number of glycopeptides with different numbers and configurations of Tn antigen. In addition, we synthesized glycopeptides based on a 40-kDa cryptosporidial antigen, a polymorphic surface glycoprotein...
with varying numbers of serine residues, to determine the reactivity with sera from *C. parvum*-infected individuals. The 40-kDa glycopeptides contained up to 23 repeating GalNAC-Serine residues, which have never been reported to be synthesized. These glycopeptides and non-glycosylated peptides were printed to generate a glycopeptide microarray for screening of sera from *C. parvum*-infected individuals for the presence of IgM and IgG antibodies. Sera from *C. parvum*-infected individuals bound to multivalent Tn antigen epitopes presented on glycopeptides, suggesting that Tn-containing glycoproteins from *C. parvum* induce immune responses upon infection. In addition, molecular differences in glycosylated peptides, such as substituting Serine for Threonine, as well as the residues that are glycosylated had a pronounced effect on reactivity. Pooled sera from individuals infected with other parasites including *Toxoplasma* and *Plasmodium* were also tested against the modified *Cryptosporidium* peptides, and some sera showed specific binding to glycopeptide epitopes. These studies reveal that specific anti-glycopeptide antibodies that recognize the Tn antigen may be applied diagnostically and in defining the roles of parasite glycoconjugates in infections. This study also highlights the successful synthesis of repeating units of GalNAC-Serine as part of a *C. parvum*-derived sequence, which has previously been thought to be too difficult to generate. This technological advance in glycopeptide synthesis will be utilized for generating a battery of glycopeptide sequences, and the current arrays will be screened with additional cohorts of *Cryptosporidium*-infected serum.

(186) *Leishmania* proteophosphoglycans (PPGs): genetic analysis of a parasite mucin-like glycoprotein implicated in several key steps in the infectious cycle

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Phosphoglycans (PPG) are abundant mucin-like glycoproteins found on the surface and secreted by the trypanosomatid protozoan parasite *Leishmania*. PPG proteins range up to 2 MDa, mostly comprised of tandemly repeated 15-17 amino acid serine-rich motifs. The peptide repeats in turn are heavily modified by substituted phosphoglycan (PG) repeating units [Gal[β1, 4]-Man(α1)-PO₂], first identified in the more abundant *Leishmania* lipophosphoglycan (LPG). Unlike LPG where the PG repeating units are attached to a GPI-core acceptor, PG repeats are attached to the PPG protein backbone through Man-PO₂-Ser linkages. While less abundant than LPG, PPGs are expressed throughout the infectious cycle, and have been proposed to carry out several unique roles independent of LPG. In the sand fly vector, PPGs form a gel-like ‘plug’ that alters sand fly feeding and parasite transmission. In the mammalian host, inoculation of the ‘plug’ and/or PPG expression in the phagolysosome may exacerbate parasite infectivity and pathology. In previous studies we used specifically LPG-deficient/ PPG-replete mutants (such as *lpg*) to unambiguously assess the unique roles of LPG across the parasite infectious cycle. Here we tackled the challenge (2 loci, very large genes, cluster >100 kb) of getting a specifically PPG-null mutant through deletion of the PPG protein backbone. PPGs are encoded by 5 genes at two loci; PPG2 on chromosome 33 and a cluster of >100 kb encoding PPG[3-4-5-1] on chromosome 35. We were able to successfully generate homozygous replacements of both loci independently (ppg-cluster or ppg2), and a double mutant lacking both the PPG cluster and PPG2 (ppg-null). Southern blot analysis suggests the true size of the PPG cluster to be 180 kb, making the ppg-cluster KO the largest chromosomal deletion generated in *Leishmania* thus far. Western blot analysis with anti-PG antisera was used to confirm the loss of PPG expression. We recently generated ‘add-backs’ restoring one or more PPG genes, and have initiated studies exploring the consequences of PPG ablation across the infectious cycle.

(187) Virulent and avirulent strains of *Toxoplasma gondii* which differ in their glycosylphosphatidyl-inositol content induce comparable biological functions in macrophages

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Glycosylphosphatidyl-inositol (GPI) from several protozoan parasites are thought to elicit a detrimental stimulation of the host innate immune system aside their main function to anchor surface proteins. Here, we analyzed the GPI biosynthesis of an avirulent *Toxoplasma gondii* type 2 strain (PTG) by metabolic radioactive labeling. We determined the biological function of individual GPI species in the PTG strain in comparison with previously characterized GPI-anchors of a virulent strain (RH). The GPI intermediates of both strains were structurally similar, however the abundance of two of six GPI intermediates was significantly reduced in the PTG strain. A side-by-side comparison of GPI-anchor content revealed that the PTG strain had only ~34% of the protein-free GPIs as well as ~70% of the GPI-anchored proteins with significantly lower rates of protein-N-glycosylation compared to the RH strain. All mature GPIs from both strains induced comparable secretion levels of TNF-α and IL-12p40, and initiated TLR4/MyD88-dependent NF-κB activation in macrophages. Taken together, these results demonstrate that PTG and RH strains differ in their GPI biosynthesis and possess significantly different GPI-anchor content, while individual GPI species of both strains induce similar biological functions in macrophages.
Galectins are evolutionary conserved family of β-galactoside binding proteins, are known to modulate interactions between cells, or cells and extracellular matrix. Galectins are ubiquitous in eukaryotic taxa found with three types: "proto", "chimera" and "tandem-repeat". Galectins mediate diverse biological processes such as development, apoptosis and tumor metastasis. More recently, their roles in immune regulation, and as pattern recognition receptors in innate immunity have been identified, although the detailed mechanisms in host-pathogen interactions and particularly on virus entry are not clearly understood. The infectious hematopoietic necrosis virus (IHNV, Novirhabdovirus, Rhabdoviridae) infects teleost fish causing infectious hematopoietic necrosis and death, with a severe impact on trout and salmon aquaculture. IHNV has a bullet-shaped virion containing a non-segmented, negative-sense, single-stranded RNA genome of approximately 11,000 nucleotides that encodes six proteins in the following order: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G), a non-structural protein (NS), and a polymerase (L). A recombinant pIHNV-EGFP plasmid strain was constructed by insertion of an EGFP ORF, fused with a copy of the untranslated region between the P and M ORFs at the 3' end, into the pIHNV backbone. In this study we examined the expression of various host response factors (galectins, membrane proteins, cytokines, viral effectors and SOCS) in fish cell lines (ZF2 from the zebrafish, and EPC from fathead minnow) upon IHNV and poly I:C challenges. The successful infection of the IHNV in both the EPC and ZFL cell lines was confirmed by the GFP-IHNV fluorescence under microscope, PCR amplification, and Western blot. The virus entry was monitored after 15 min to 30 min of virus exposure using immunofluorescence staining. RNA and proteins are extracted from the challenged ZFL and EPC cells at different time points within 72 hours. A gradual increase of galectin (Drgal1, Drgal3, Drgal-9) expression at 6 to 8 hours of infection was observed, which returned to the basal level at 24 hours when compared to that of the uninfected controls. Expression of cytokines and antiviral effectors (Mx a,b,c) was also modulated significantly by the infectious challenge. Ongoing studies are aimed at the identification of the entry mechanisms, particularly those that may be mediated by secreted galectins, and the host responses that result from galectin-virus interactions [Supported by grant 5R01GM070589-06 from the National Institutes of Health to GRV, and the Department of Biotechnology, New Delhi, India, to CR].

Galectins are a conserved family of carbohydrate-binding proteins involved in a wide variety of cellular processes, such as cell-cell interactions, tumor proliferation and metastasis, and regulation of innate and adaptive immune responses. Based on distinctive structural features, galectins are classified into the "proto", "chimera" and "tandem-repeat" types. Our prior studies have shown that all three categories are expressed in zebrafish (Danio rerio), and suggested that DrGal1, DrGal3 and DrGal9 play a key role during experimental infection of zebrafish cell lines with infectious hematopoietic necrosis virus (IHNV). IHNV, an economically important pathogen of both wild and farmed salmonids, is a negative-sense single-stranded virus belonging to the genus Novirhabdovirus. The lack of standardized tools for the rigorous detection and quantification of IHNV viral particles in vitro, and commercial antibodies for use in standard immunoassays has seriously hindered progress toward the resolution of this problem. Thus, the development of tools and techniques that will enable the optimization of the IHNV-zebrafish infection model for the mechanistic study of the IHNV infection process and the subsequent host immune response is of great interest. In this study, we designed a protocol for production and purification of the viral G (glycoprotein) and M (matrix) proteins of IHNV in recombinant bacteria, and optimized their use for the production of polyclonal antibodies, that will enable us to visualize potential interaction between galectins and IHNV particles. We also optimized a protocol for the purification of IHNV by high-speed centrifugation and subsequent labeling with an amine reactive ester of biotin. The establishment of protocols to efficiently produce IHNV proteins and highly specific polyclonal antibodies will improve the use of standard assays for IHNV detection and purification, and facilitate drug discovery through high-throughput screening assays [Supported by grant 5R01GM070589-06 from the National Institutes of Health to GRV, and the Department of Biotechnology, New Delhi, India, to CR; NGM is supported by grant 5T32AI095190-02; RK is supported by the NSF Expert Program].

Chemoenzymatic-tagging of whole influenza virus for direct detection on glycans microarrays
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Infections from influenza type A viruses are a major concern to the human population and the poultry industry. Viral receptor specificity
is well documented as a determinant in the host range of the virus and, acquisition of human type receptor specificity is established as a risk factor for pandemic virus emerging from animal populations. Specificity is determined by the virus’ hemagglutinin protein’s ability to bind human type (α2-6) or avian type (α2-3) sialic acid receptors located on tissue glycoproteins of host airways. Receptor specificity is typically assessed using glycan microarrays or plate assays in which, the virus is detected with antibodies or can be directly labeled using chemical tagging. For new influenza isolates, preparation of a suitable antibody is required, and chemical labeling requires large scale growth and purification of the virus. Antiserum raised against whole virus can also contain antibodies cross-reactive with glycans that could interfere with certain assays. We have developed a simple rapid method to introduce a biotin tag to whole virus using a chemo-enzymatic approach. We take advantage of the fact that glycans of the surface glycoproteins of influenza grown in eggs or cell culture are terminated in galactose. Brief reaction with galactose oxidase introduces an aldehyde at the 6C-position of galactose, which can then be efficiently tagged using aniline catalyzed conjugation of biotin using commercial biotin-oxymine (Galactose-Aniline-Ligation, or GAL). GAL labeled viruses bear fully functional hemagglutinin and neuraminidase proteins, which can be assessed for receptor specificity on a custom sialoside glycan array bearing relevant avian and human type sialosides. Comparisons to antibody detection and NHS-based chemical labeling against whole H1N1, H3N2 and H5N1 influenza subtypes show equivalent or superior results with our direct labeling method. The GAL labeling procedure of influenza virus is a rapid, cost-effective and facile method that can enhance the study of virus glycan receptor specificity. (Funded by a contract from the Center for Disease Control).

(191) Polysialic acid is a Potent Inducer of Antigen Cross-presentation and Antigen-Specific Cytotoxic T cell Activation in Human and Murine Cells
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Polysialic acid (also known as colominic acid), a homopolymer of N-acetylneuraminic acid with (2-8) ketosidic linkages, can be produced by chemical and enzymatic methods from strains of Escherichia coli possessing the K1 antigen. Using an immature murine dendritic cell (DC) line and a murine cytotoxic T cell (Tc) hybirdoma cell line that can be activated specifically by ovalbumin presented as antigen by the DC MHCI molecules, we found that polysialic acid is a potent inducer of antigen cross-presentation and the subsequent activation of antigen-specific cytotoxic T cells in tissue cultures. Priming immature murine DC line cells or primary human DCs with polysialic acid, subsequently pulsing these primed DCs with tumor cell lysate and then exposing these DCs to the corresponding Tc cells extracted from mouse spleen or human peripheral blood similarly activates differentiation of the homologous antigen-specific Tc cells. Thus prepared Tc cells killed significantly more human lung cancer and murine melanoma cell line cells, from which the priming tumor cell lysates had been prepared, than did Tc cells treated by DCs that had not been primed with polysialic acid. Using endocytosis inhibitors chlorpromazine, nystatin and 5-(N,N-dimethyl)amiloride (that block clathrin-mediated endocytosis, lipid-raft-caveolae-mediated endocytosis and pinocytosis respectively), we found that polysialic acid primed antigen uptake by dendritic cells is dependent on both clathrin-mediated endocytosis and lipid-raft-caveolae-mediated endocytosis, but not the pinocytosis pathway. Inhibition by Brefeldin A indicates that antigen taken up by the polysialic acid primed DCs is transported from the ER to the Golgi before being cross-presented to activate Tc cells. These data suggest that polysialic acid is a potentially potent adjuvant for cancer vaccines.

(192) The commensal glycoantigen PSA inhibits inflammation through a novel mechanism of T cell-T cell interaction
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Asthma and airway inflammatory disease has become the leading cause for ER visits in the US, a four-decade trend linked to the increasing sterility of western lifestyles and a proposed lack of early microbial antigen exposure. Here, we demonstrate that gastrointestinal contact with the Bacteroides fragilis glycoantigen PSA educates the peripheral immune system to limit the degree of inflammation. Effective immune responses upon re-exposure to an antigen is mediated through memory T cells (Tmem), whereas regulatory T cells (Treg) play key roles in suppressing immune responses, generally through the secretion of immunosuppressive cytokines, principally IL-10. We show that PSA exposure increases a population of antigen experienced CD45Rb+ Tmem cells, and when these Tmem cells interact with FoxP3+ Tregs, the anti-inflammatory activity of Tregs, as measured by IL-10 production, is synergistically amplified far above their response in isolation. We propose a novel pathway of T cell-crosstalk in which commensal microbiota-driven Tmem cells synergistically promote IL-10 secretion in resident FoxP3+ Tregs to dampen an inflammatory response. These findings expose a pathway that provides a mechanistic framework for the hygiene hypothesis where communication between Tmem and Treg cells connects the microbiota with non-specific peripheral immune quiescence resulting in reduced susceptibility to inflammation.

(193) IgG Fc glycosylation changes through lifetime
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Glycans attached to the conserved Fc N-glycosylation site of IgG molecule strongly affect its binding to different Fc receptors and
modulate many other aspects of the immune system. IgG Fc glycosylation modulates inflammation and through promotion or suppression of inflammation it may significantly contribute to the process of biological aging. By analysing IgG glycosylation in 5117 individuals from four European populations we have revealed very extensive and complex changes of IgG glycosylation with age. The index composed of only three IgG glycans (FA2B, FA2G2 and FA2BG2) that considerably change with age explained 58% of variance in chronological age. The remaining variance in these glycans strongly correlated with physiological parameters associated with biological age. Thus IgG glycosylation appears to be closely linked with both chronological and biological age. Considering the important role of IgG glycans in inflammation, and since the observed changes with age promote inflammation, changes in IgG glycosylation may actually represent a factor contributing to aging.

**194. The N-glycome of dromedary camel serum and IgG**

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Dromedary camels are an important agricultural species and are of biomedical interest since they produce single chain antibodies. However, little is known about the glycosylation of camel serum and IgG. In this study the serum and IgG N-glycome from two different dromedary camel breeds were fully analyzed by a combination of complementary chromatography and mass spectrometry based approaches. N-glycans from camel serum and IgG were released, purified and fluorescently labeled on a high-throughput robotic platform and analyzed by HILIC-UPLC-FLD, exoglycosidase digestions and LC-MS. Thirty five distinct neutral and sialylated N-glycan structures were identified in camel IgG and forty eight structures in camel serum. Camel IgGs represent an equal mix of core-fucosylated and non-core-fucosylated N-glycans and contain alpha-linked galactose as well as both N-glycolyneuraminic acid and N-acetyleneuraminic acid. IgG N-glycans dominate the glycan profile of camel serum; interestingly supplementary O-acetylated glycans were also identified in serum. While the relative glycan abundances were similar within the same breed, differences were observed between the two breeds. The comprehensive analysis and annotation of the serum and IgG N-glycome is important given its wide applications, including biomarker studies, biopharmaceutical development as well as genome wide association studies.

**195. IgG and total plasma glycome during systemic inflammatory response**

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Controlling the inflammatory cascade represents a great challenge, due to its complexity and individual physiological differences.

Although known to have a great impact on protein functions, changes of glycosylation in acute inflammation have not been extensively studied due to absence of a good human model. The aim of this study was to follow intraindividual changes of total plasma and IgG glycans in 107 patients, prior and during the early course of systemic inflammation caused by cardiac surgery. Both IgG and plasma protein linked glycans showed prominent, dynamic changes. Contrary to the plasma glycome, pattern of IgG glycosylation changes was rather different between individuals. Correlation was observed between basal levels of IgG fucosylation and the rate of its change with the severity of the inflammatory response, suggesting prognostic potential of glycosylation. These results imply new potential targets for controlling the inflammatory response and must be considered when immunomodulating therapies are subjected.

**196. Targeted modification of protein N-glycosylation results in a spontaneous CVID-like condition associated with anti-lymphocyte autoimmunity**

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Common variable immunodeficiency (CVID), the most frequent symptomatic primary immune deficiency in humans, is a heterogeneous group of immunologic disorders effecting about 1 in 30,000. Although a clear disease etiology remains elusive, a common characteristic of CVID is deficient IgG antibody production in response to infection or vaccination. Many patients also exhibit symptoms of abnormal T cell function, including an apparent lack of naïve T cells which correlates with clinical severity. In our present study, we describe one of the first animal models of spontaneous CVID that incorporates aspects of both humoral and T cell dysfunction. The CVID mice exhibit characteristic deficiencies in IgG responses to both protein and polysaccharide vaccines. Interestingly, the immunodeficiency is associated with decreased T cell activity due to a persistent autoimmune response depleting naïve T cells, which appears to be mechanistically linked to changes in erythrocyte surface N-glycosylation. The N-glycosylation dependent auto-epitopes that emerge on erythrocytes were found to cross-react with naïve T cells independent of changes to the latter’s glycosylation profile. While the use of Ig replacement therapy (i.e., IVIg) and antibiotics has reduced the number of infections in CVID, the major inflammatory complications such as lung disease, cancer, and autoimmunity still develop; thus our findings provide potential insight into human CVID beyond hypogammaglobulinemia by addressing the mechanisms that may contribute to the more broad defects in adaptive immunity linked to clinical severity. (This work is supported by grants OD004225 and GM082916 to BA Cobb and with assistance from CFG Core H)

**197. Large-scale preparation of Asn-glycopeptide carrying plant antigenic N-glycan and the immunomodulatory activity on human monocyte derived dendritic cells**

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It has been believed that the highly antigenic N-glycans bearing \( \beta 1-2 \) xylosyl and/or \( \alpha 1-3 \) fucosyl residues are ubiquitously expressed in higher plants. As for the involvement of plant antigenic N-glycans in Japanese cedar pollinosis, we have reported that IgE against Japanese cedar allergen, Cry j1, in about 25% of patients with the pollinosis recognizes the antigenic N-glycans, but the clinical relevance of plant antigenic N-glycan specific antibodies has not been still revealed. On the other hand, we found that the free antigenic N-glycans suppresses the production of IL-4 from Cry j1 specific Th2 cells, suggesting that plant antigenic N-glycans can be used as leading compound in developing immuno-pharmaceuticals. In this study, to prepare a large amounts of non-labeled immunoreactive Asn-glycopeptides bearing structurally homologous plant antigenic N-glycans, we developed a new preparative procedure using a combination of gel-filtration and hydrophilic partitioning method. Finally, we obtained about 103 mg of Asn-glycopeptide bearing the antigenic N-glycans from 1.7 kg of defatted powder of Ginkgo biloba seeds. Second, in order to examine the immunomodulatory activity of plant antigenic N-glycans, we prepared a glycopolymer that Asn-glycopeptide was coupled to poly-\( \gamma \)-L-glutamic acid (\( \gamma \)-PGA). Amino acid composition analysis showed that N-glycans were incorporated into \( \gamma \)-PGA about ten mol %. Lastly, human immature DC (iDC) were prepared by culturing human CD14 monocytes in the presence of IL-4 and granulocyte-macrophage-stimulating factor for six days. The iDC were stimulated with \( \gamma \)-PGA or glycopolymer for 48 hours, and analyzed the DC maturation markers (CD80, CD86, and HLA-DR) by flow cytometry. The glycopolymer inhibited the DC maturation induced with \( \gamma \)-PGA. These results suggest that the glycopolymer having plant antigenic N-glycans suppresses T cells activation via DC maturation. To reveal the structural specificity in inhibition of DC maturation, we are now preparing a wide variety of glycopolymer using by antigenic N-glycans and non-antigenic N-glycans.

References

(198) Critical role of the C-type lectin receptor DCIR in cerebral malaria pathogenesis
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Malaria is a major global threat, resulting in more than 200 million clinical cases and 655,000 deaths per year. The most severe complication of this disease is cerebral malaria (CM), accompanied by impaired consciousness, coma and generalized convulsions. An experimental model of CM that shares characteristics with the human disease is infection of C57BL/6 mice with Plasmodium berghei ANKA (PbA). While the contribution of T cells to CM development has been investigated intensively, still little is known about the impact of innate immunity on CM pathogenesis. Antigen-presenting cells such as dendritic cells sense pathogens via pattern recognition receptors and play a pivotal role in the initiation of adaptive immune responses during infections. Myeloid C-type lectin receptors (CLRs) represent a family of pattern recognition receptors predominantly expressed by antigen-presenting cells. CLRs recognize carbohydrate structures on pathogens and self-antigens often in a Ca2+-dependent manner. In this study, we analyzed the function of the CLR Dendritic Cell Immunoreceptor (DCIR) in the pathogenesis of CM. DCIR is mainly expressed by dendritic cells, but also by monocytes, macrophages, and B cells. We demonstrate here that DCIR is crucial for CM induction as DCIR-deficient mice were highly protected from CM in the murine PbA infection model. In agreement with the reduced CM incidence, DCIR− mice exhibited a reduced CD8+ T cell sequestration in the brain and ameliorated brain inflammation. DCIR deficiency also led to decreased serum TNF-α levels, affected CD4+ and CD8+ T cell priming in spleen and modulated T cell effector functions during the course of malaria. In conclusion, this study demonstrates a pivotal role for DCIR in CM development and highlights the importance of CLRs as pattern recognition receptors in infectious diseases.

(199) Role of Galectin-3 In Exosome Biogenesis and Secretion In Dendritic Cells
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Exosomes are small vesicles (50-100 nm) secreted by different cell types. The exosomal secretion pathway is primed by intraluminal vesicles (ILVs) budding into the late endosomes to form multivesicular bodies (MVBs), which then fuse with the plasma membrane releasing in result of ILVs to the extracellular space. The exosomal component proteins include the ESCRT (endosomal sorting complex required for transport) family members (Alix, Tsg101), tetraspanins family members (CD9, CD63, CD81), membrane trafficking members (Rab5, Rab11b), and galectin-3. Exosomes function in cell-to-cell communication, antigen presentation, Oocyte differentiation, and tumor progression. However, the mechanism of exosome secretion and biogenesis is still unclear. Galectin-3 has been identified as a component of dendritic cell (DC)-derived exosomes by proteomic analysis. As galectin-3 can be associated with Alix in a number of cell types, we hypothesized that galectin-3 may participate in exosome biogenesis through interacting with Alix. Exosomes were purified from gal3+/+ or gal3−/− BMDCs by ultracentrifuge and subjected to immunoblotting analyses, semi-quantitative fluorescence-activated cell scanning (FACS)-based assay, and immuno-EM to identify exosomal phenotype. Finally, MVBs in gal3+/+ and gal3−/− BMDCs were studied by immunofluorescence staining and immune-EM. Immunofluorescence staining showed that galectin-3 is colocalized with Alix and CD63 in BMDCs. Immuno-EM and immunoblotting analyses showed that galectin-3 is expressed on the surface as well as inside of DC-derived exosomes. By comparing exosomes collected from BMDCs, we further discovered that gal3+/−BMDCs secreted more exosomes than gal3+/+ BMDCs. Immunoblotting analysis of the
proteins with different levels of protein components compared to those from gal3+/+ BMDCs. These results suggest that galectin-3 may play a role in regulating exosome biogenesis and secretion.

(200) Desialylation of airway epithelial cells during influenza infection enhances pneumococcal adhesion via galectin binding
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Galectins are a family of soluble β-galactoside-binding proteins that are synthesized in the cytosol and may carry out their biological roles in the nuclear compartment, at the cell surface, and the extracellular space. They are classified into three major structural types: (i) proto-type, (ii) chimera-type, and (iii) tandem-repeat-type galectins. Galectins are expressed in various cell types, including neutrophils, macrophages, dendritic cells, B and T cells. Further, a substantial body of evidence supports their critical roles in immune homeostasis, and recent evidence suggests their roles in the lung innate immune response to pneumococcal infection, although the mechanisms remain largely unknown. Recently, the explosive onset of pandemic influenza has revealed a need to gain further insight into the pulmonary innate immune response to invading microbes. Pneumococcal exposure following influenza virus infection is a major trigger of patient mortality. In this study, we analyzed the patterns of galectin expression and secretion in lung tissue and in bronchoalveolar lavage from mice that had been exposed to influenza (PR8) and S. pneumoniae (Sp3). Furthermore, we examined the mechanisms by which galectin interactions with endogenous (“self”) and exogenous (“non-self”) lactosamine containing glycans control innate immune responses to viral and bacterial infections in the lung. Influenza infection, more specifically influenza neuraminidase, triggers an upregulation of galectin1 and galectin3. Exposure of airway cell lines to PR-8 increases adhesion of Sp3, suggesting that it attenuates the resistance of the host to the secondary bacterial infection S. pneumoniae. Moreover, PR8-and Sp3-challenged galectin3 knockout mice exhibited more moderate symptoms and higher survival rate than wild type mice, revealing the role of galectin3 in activation of pro-inflammatory pathways.

(201) Recognition of mannose and sialic acid containing glycans mediate neutrophil activation
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For a long time, neutrophils were considered to be just soldiers in the front line of immune response, cells prepared to attack and to die rapidly. The scenario around this idea is rapidly changing, since the discovery of sophisticated immune mechanisms involving neutrophils. Recent literature data attribute new functions to neutrophils, essentially related to their capacity of producing cytokines and communicating with other cells through direct contact, granules or DNA networks releasing. In this context, neutrophils are proposed to participate in protection against intracellular pathogens such as viruses, mycobacteria and protozoa parasites. Although several lectins are reported to activate neutrophils, the concerning studies did not approach the novel functions attributed to these cells, nor tried to identify which surface glycans are involved in triggering neutrophil activation. By taking advantage of three exogenous lectins with distinct sugar recognition specificities, this study aimed to determine the neutrophil responses to the stimulation with those lectins. Two of them were obtained from Artocarpus heterophyllus: ArtinM that recognizes the trimannoside core of N-glycans, and Jacalin that recognizes the Galβ1,3GalNAc core of O-glycans. The third one is a micromere protein (TgMIC1) from Toxoplasma gondii, which binds to sialylated glycans. Firstly, we investigated the neutrophil migration induced by the lectins when injected into the peritoneal cavity of BALB/c mice; a positive response was provided by ArtinM and TgMIC1, but not by Jacalin. The lectins capacity of activating human neutrophils was evaluated in vitro. Again, stimulus with ArtinM or TgMIC1, but not by Jacalin, has induced neutrophils to produce IL-8 and to degranulate, as observed by assaysing the cell supernatant for the cytokine levels and for detection of myeloperoxidase and elastase activities. In addition, ArtinM and TgMIC1 were able to enhance the phagocytic capacity of human neutrophils toward fluorescent beads. Finally, Tg MIC1 induces formation of neutrophil extracellular traps (NETs). We conclude that ArtinM and TgMIC1 lectins are able to activate neutrophils, manifested by increased IL-8 production, cell degranulation, phagocytosis and NET formation. Mannose and sialic acid containing glycans seems to be good targets for triggering neutrophil activation and possibly for promoting pathogens elimination.

(202) N-glycosylation deficiency caused ICAM-1 reduction correlates with impaired innate immunity
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Congenital Disorders of Glycosylation (CDGs) result from mutations in various N-glycosylation genes. The most common type, PMM2-CDG (CDG-Ia), is due to deficient phosphomannomutase-2 (Man-6-P → Man-1-P). Many patients die from recurrent infections,
but the mechanism is unknown. We found that CDG patient fibroblasts have less intercellular adhesion molecule-1 (ICAM-1), and because of its role in innate immune response, we hypothesized that its reduction might explain recurrent infections. In support of this, tunicamycin blocks TNF-α induced ICAM-1 production, suggesting that many CDG cells may show poor ICAM-1 response. We, therefore, investigated a mouse model of MPI-CDG (CDG-Ib), deficient in phosphomannose isomerase, (Fru-6-P → Man-6-P). We challenged MPI-deficient mice with an intraperitoneal injection of pro-inflammatory mediator (Zymosan) and found decreased neutrophil extravasation compared to controls. Immunohistochemistry of mesenteries showed attenuated neutrophil egress, presumably due to poor ICAM-1 response to acute peritonitis. Since CDG-Ib patients and their cells improve glycosylation when given mannose, we provided MPI mice with mannose-supplemented water for 7 days. This increased ICAM-1 expression on mesenteric endothelial cells of Zymosan-challenged mice and enhanced transendothelial migration of neutrophils compared to untreated controls. Attenuated inflammatory response in glycosylation-deficient mice may result from a failure to increase ICAM-1 on the vascular endothelial surface and may help explain high mortality in patients. The work was supported by the Rocket Fund & NIH R01 DK55615.

(203) B cell development is possibly influenced by the circulatory pool of free ST6Gal-1 sialyltransferase from the liver

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A role for the ST6Gal-1 sialyltransferase in B lymphocyte development and function is well established. Animals with global ST6Gal-1 deficiency have impaired humoral responses, impaired B cell development, reduced B Cell Receptor (BCR) activation, decreased marginal zone B cell population in the spleen, and reduced homing of mature B cells to bone marrow. It has been assumed that these defects are due to the inability of B lineage cells to express ST6Gal-1. The P1 promoter, one of six distinct transcriptional promoters regulating St6gal1, drives ST6Gal-1 expression in the liver. A sizable pool of soluble, extracellular ST6Gal-1 is present in systemic circulation. Disabling P1 strikingly decreases the circulatory levels of ST6Gal-1 while leaving intact St6gal1 transcription from other promoters in non-hepatic tissues. P1 is not utilized in B-lineage cells. However, St6gal1-dP1 mice with disabled P1 have defects in the B lineage compartment similar to the global ST6Gal-1 deficient mice, St6gal1-KO. The St6gal1-dP1 mouse has altered B cell development, including a 2-fold reduction in splenic marginal zone (MZ) B cells and a 2-fold increase in transitional type (T1) splenic cells, despite an outwardly normal humoral response when challenged by the test hapten NP-[4-d-hydroxy-3-nitrophenyl]acetyl] with identical kinetics and magnitude of anti-NP antibody production. Together, our observation suggests that a subset of the B cell defects in the global ST6Gal-1 deficiency may be due to a decreased pool of circulatory sialyltransferase originating from the liver. (Funded by NIH Program of Excellence in Glycosciences P01HL107146 and NIH R01AI56082)

(204) Glycan “Node” Analysis for Detecting and Monitoring Cancer

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Dysregulated glycotransferase enzymes in cancer cells produce aberrant glycans-some of which can help facilitate metastases. Within a cell, individual glycotransferases promiscuously help construct dozens of unique glycan structures, making it difficult to comprehensively track their activity in biospecimens-especially where they are absent or inactive. An approach is described to deconstruct glycans in whole biospecimens then analytically pool together resulting monosaccharide-and-linkage-specific degradation products (glycan "nodes") that directly represent the activities of specific glycotransferases. To implement this concept a reproducible, relative quantitation-based glycan methylation analysis methodology was developed that simultaneously captures information from N-, O-, and lipid linked glycans and is compatible with whole biofluids and homogenized tissues; in total over 30 different glycan nodes are detectable per GC-MS run. Numerous non-liver organ cancers are known to produce induction of abnormally glycosylated serum proteins. Thus following analytical validation in blood plasma the technique was applied to a cohort of 59 lung cancer patient plasma samples and age/ gender/ smoking-status matched non-neoplastic controls from the INCO Copernicus lung cancer case-control study to gauge the clinical utility of the approach towards detection of lung cancer. Ten smoking-independent glycan node ratios were found that detect lung cancer with individual ROC c-statistics ranging from 0.76-0.88. Two glycan nodes provided novel evidence for altered ST6Gal-I and GnT-IV glycotransferase activities in lung cancer patients. In summary, a conceptually novel approach to the analysis of glycans in unfractionated human biospecimens has been developed that, upon clinical validation for specific applications, may provide diagnostic and/or predictive information in glycan-altering diseases.

(205) Global gene expression studies for identification of signaling pathways leading to Sclerotium rolfsii lectin induced cell death in human colon cancer cells

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Sclerotium rolfsii lec (SRL) has exquisite binding specificity to Thomsen-Friedenreich antigen and associated glycans expressed on cancer cells. Earlier studies have shown that SRL exhibits strong receptor mediated antiproliferative effect towards human colon cancer HT 29 cells by inducing apoptosis, involving participation of extrinsic and intrinsic pathways. SRL at sub toxic concentrations completely inhibits tumour growth in vivo (Shashikala et al. 2012). In this study, we have investigated the signaling pathways elicited in
response to SRL treatment in HT 29 cells that lead to cellular apoptosis. HT29 cells treated without or with SRL were harvested at different time points of 2, 4, 8 12, 24, 36 and 48h, and gene expression microarray analysis was performed using Agilent’s SurePrint G3 Human Gene Expression 8X60K Microarray kit. Results reveal that several hundred entities were differentially regulated upon lectin treatment at all-time points with a fold change value of ≥2 and p value ≤ 0.05. Pathway analysis revealed that the MAP Kinase signaling pathway is affected as early as 2h while cell cycle and the DNA damage response pathways are most significantly affected at later time points. SRL treatment led to immediate over expression of the Jun transcription factor, known to be involved in apoptosis. The early time point’s miRNA expression study showed that very few miRNAs to be differentially regulated at 2 and 4 h after treatment. However, a significant change in differentially expressed miRNAs was noticed at 12h with the miRNA target list significantly overlapping with the differential gene expression list. The present study suggests that the interaction of SRL with HT29 cells triggers apoptosis in these cancer cells by affecting multiple pathways, like the cell cycle pathway, concurrently inducing DNA damage, initiated through the MAP kinase pathway and mediated by the transcription factor JUN. The findings will enable to exploit SRL for its possible application in cancer diagnostics and also as a targeted drug for cancer therapies. The TF-antigen binding lectin from Sclerotium rolfsii inhibits tumour cell growth by induction of apoptosis.

Reference

(206) Sugar-azide Metabolic Labeling to Identify Lung Cancer Glycoprotein Biomarker Candidates
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Non-Small Cell Lung Cancer (NSCLC) represents 85% of all lung cancers with an average 5 year life expectancy of 15-20%. Efforts to identify markers that will predict the course of these tumors, as well as response to therapeutics, have been difficult due to heterogeneity in the cancer progression and variation in the environmental factors that contribute to them. Tissue inflammation and the promotion of remodeling programs from chronic cellular insult are common features in the pathogenesis of NSCLC. Central to these processes is transforming growth factor beta (TGF-beta), a pleiotropic cytokine hypothesized to promote a phenotype switch in cancer cells resulting in modulation of adhesion molecules, cytoskeletal reorganization, and the production of extracellular matrix remodeling enzymes. In an attempt to better define the glycoproteins involved in this process, a TGF-beta inducible H358 bronchoalveolar carcinoma cell line has been used in metabolic labeling experiments with the azide-modified sugar analog of sialic acid, N-acetylamannosamine-azide (ManNAcAz), and N-acetylgalactosamine (GalNAcAz). Following doxycycline-inducible expression of TGF-beta or vector control, the incorporation of the sugar azide derivatives into proteins in media or cell lysates modified with N-or O-linked glycans, glycosaminoglycans or O-GlcNAc conjugates were determined using an optimized alkyne-bead capture method, on-bead trypsin digestion and LC-MS/ MS. In general, protein numbers from cells treated with either analog sugar and induced with doxycycline ranged from 100-150 hits for cell lysate fractions, and include differential capture of cadherins, adhesion molecules and integrins. In addition, an abundance of integrin-interacting scaffolding proteins like CDCP-1, neuropilin-1/2, tetraspanins and basigin were identified, highlighting a class of proteins that are heavily glycosylated with both N- and O-linked glycans. Secretome fractions also showed enriched selection of glycoproteins in treated groups as evidenced by the detection of extracellular-matrix proteins such as metalloproteinases, SPARC, and dystroglycan. Finally, peptide-N-glycosidase-F digestion of glycopeptide-bound alkyne beads was used as an initial screen to evaluate the release of N-linked glycan peptides bearing the ManNAcAz analog between experimental groups. Preliminary results demonstrate an increased identification of peptides harboring the N-X-S/ T consensus sequence and the expected deamidated Asn. This H358 cell system is also being used in direct glycopeptide analysis using HCD-PD-ETD detection, in combination with SILAC quantitation.

(207) DNA hypomethylation at the transcription start site of MGAT3 correlates with increased expression in ovarian cancer cells
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Advanced stage epithelial ovarian cancer has a poor survival rate of less than 30%. Although prognosis improves significantly with early diagnosis, the lack of a reliable and sensitive biomarker as well as limited understanding of this heterogeneous disease hinders detection of ovarian cancers at an early stage. In an effort to identify surface markers on ovarian cancer cells by LC-MS/ MS, we have previously detected complex glycan structures comprising a unique bisecting GlcNAc motif exclusively on serous ovarian cancer cells. Bisecting GlcNAc on N-glycosylated proteins is a product of the beta-1,4-N-acetylgalactosaminyltransferase 3, MGAT3. In this study, gene and protein expression analysis support our recent findings of elevated MGAT3 expression in ovarian cancer cell lines when compared with normal human ovarian surface epithelial (HOSE) cells. Interestingly, MGAT3 and bisecting GlcNAc expression can be induced in HOSE cell lines by inhibition of DNA methyltransferases using the compound 5-aza. We analyzed DNA methylation in the MGAT3 promoter region, which contains a large CpG island with 204 CpG dinucleotides located -600/ +800bp relative to the transcription start site (TSS). Our bisulfite sequencing result revealed that increased MGAT3 expression in ovarian cancer cells coincides with a significant reduction of DNA methylation. We have analyzed 37 CpGs located -200/ +30bp relative to the TSS of MGAT3 and observed 19 and 36% methylation in two serous ovarian cancer cell lines compared with 60 and 71% methylation in two HOSE cells.
Growth factor-induced tyrosine kinase receptor (RTK) activation in pancreatic cancer cell survival responses has become a strategic molecular-targeting therapeutic intent. Several growth factors in the RTK family are overexpressed during the progression of pancreatic cancer. When a growth factor binds to its RTK, such as nerve growth factor for Trk, epidermal growth factor, and insulin, we have discovered that the receptor undergoes a conformational change to activate matrix metalloproteinase-9 (MMP-9) which induces Neu1 sialidase (Neu1). Both MMP-9 and Neu1 form a complex with the RTKs at the ectodomain. Activated Neu1 hydrolyzes α-2,3-sialyl residues on the receptor, enabling removal of steric hindrance to receptor association and cellular signaling. Oseltamivir phosphate (OP, Tamiflu) specifically inhibits Neu1 sialidase activity associated with growth factor-induced receptor activation. Preclinical data focused on directly targeting and inhibiting Neu1 as the key central enzyme within our newly discovered receptor signaling platform provide the evidence for a potential Tamiflu therapy in the prevention of tumor growth and metastatic spread in heterotopic xenografts of human MiaPaCa-2 tumors growing in RAGxCγy double mutant mice. The encapsulation vehicle allows for the highest concentration of the drug to be administered in a sustained manner at the tumor site. OP cylinder was developed using poly (D, L-lactic-co-glycolic acid) (PLGA) as an encapsulation polymer. PLGA is degraded through bulk erosion, releasing OP over an extended period of time. Success with a single-layered cylinder led to the development of a double-layered cylinder, containing OP in the outer layer and chemo-gemcitabine (GEM) in the inner layer. It is proposed that OP will sensitize the tumor to GEM. Both single-layered and double-layered cylinders were shown to release OP and GEM respectively over 30 days. Implanted single-layered 20 mg OP cylinders arrested tumor growth for one month with a marked decrease in tumor neovascularization. A novel anti-cancer role of Tamiflu is proposed as an alternate promising therapeutic cancer drug targeting Neu1 sialidase in the prevention of human pancreatic cancer. Research supported by a NSERC grants to MRS and RN, private-sector donor "Josefowitz Family" to MRS, NSERC Canada Graduate Scholarship to MH and CHIR Doctoral Award to SA.

(208) Therapeutic targeting Neu1 sialidase with Tamiflu disables cancer cell survival in acquired chemo-resistance of human pancreatic cancer
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Resistance to drug therapy, along with high rates of metastasis, contributes to the low survival rate of patients diagnosed with pancreatic cancer. We have reported that Neu1 sialidase is an essential enzyme which acts through a common receptor level signaling platform on the cell surface to regulate the activation of a number of glycosylated receptors important in cancer. An alternate therapeutic treatment of human pancreatic cancer by targeting Neu1 sialidase with Tamiflu was examined in acquired chemo-resistant PANC-1 cells against cisplatin and gemcitabine. Its efficacy to overcome their intrinsic resistance to chemotherapeutics and metastasis was evaluated. Microscopic imaging, immunocytochemistry and cell viability WST assays were used to evaluate cell survival, morphologic changes and expression levels of E- and VE-cadherin before and after Tamiflu treatment in established cisplatin and gemcitabine resistant PANC-1 human pancreatic cancer cells. Tamiflu dose-dependently overcomes the chemo-resistance of PANC-1 against cisplatin, gemcitabine or in combination, and disables the cancer cell survival mechanism(s). Tamiflu also reverses the epithelial-mesenchymal transition (EMT) characteristic of E-to N-cadherin phenotypic changes associated with resistance to drug therapy. Therapeutic targeting Neu1 sialidase with Tamiflu at the growth factor receptor level disables the intrinsic signaling platform for cancer cell survival in acquired chemo-resistance of human pancreatic cancer. The findings provide evidence for Tamiflu as a potential therapeutic of pancreatic cancer resistant to drug therapy. Research supported by a NSERC grant and private-sector donor "Josefowitz Family" to MRS and CHIR Doctoral Award: Banting and Best Canada Graduate Scholarship to SA.

(209) Encapsulation of oseltamivir phosphate and gemcitabine in poly (D, L-lactic-co-glycolic acid) polymer as a delivery vehicle in the treatment of pancreatic cancer in heterotopic xenografts of tumors growing in RAGxCγy double mutant mice
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Although glioma cells cultured in vitro are responsive to several anti-cancer drugs, it is very difficult to treat glioma in vivo due to blood-brain barrier (BBB). Chemotherapeutics injected to blood circulation do not pass from blood circulation to brain stroma due to...
tight junctions between endothelial cells. In the previous study, we identified a carbohydrate mimetic peptide, designated as IF7, that home tumor vasculature (Hatakeyama et al. 2011). IF7 binds to annexin A1, which is expressed specifically on the endothelial cell surface of malignant tumors. We showed that IF7 conjugated anti-cancer drug SN38 (IF7-SN38) suppressed growth of colon, melanoma, breast, prostate and lung tumor models in the mouse. Since IF7 was thought to be transported across endothelial cells through transcytotic pathway, we hypothesized that IF7 can deliver anti-cancer drug to brain stroma overcoming BBB. In this study, we produced glioma tumors in mouse brains by intracranial injection of C6 cells (Suzuki-Anekoji et al. 2011). To determine targeting of IF7 peptide to C6 glioma tumors, Alexa 488-tagged IF7 (IF7-A488) was injected intravenously. IF7-A488 targeted tumor vasculature within minutes, penetrated through endothelial cell wall and spread to the glioma sphere, while the control peptide conjugated RQ7-A488 did not. We injected luciferase-expressing C6 (C6-Luc) cells intracranially into mouse brains, and monitored growth of C6-Luc tumor by Xenogen IVIS imager. Daily intravenous injection of IF7-SN38 reduced size of C6-Luc glioma with minimum effective dose at 5.8 mg/kg, whereas as much as 95 mg/kg of non-targeted SN-38 was required to suppress subcutaneous tumors (Meyer-Losic et al. 2008). To compare targeting efficacy of IF7-SN38 between brain tumor and subcutaneous tumor, two B16-Luc tumors, one in the brain and another under the skin, were generated in a mouse. Intravenous injection of IF7-SN38 suppressed growth of these tumors with comparable efficiency regardless of their locations, demonstrating the efficacy of IF7-SN38 in overcoming BBB. These results strongly encourage clinical application of IF7-SN38 to glioma tumors. Supported by NIH grants CA33859.

References

(211) Caveolin-1 up-regulates integrin α6-sialylation to promote integrin α5β1-dependent hepatocarcinoma cell adhesion

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Caveolin-1 is a major structural protein of caveolae and plays important functions in tumorigenesis and development. Our previous studies revealed that caveolin-1 promoted cell invasion by upregulating the glycosylation of matrix metalloproteinase inducer CD147 of mouse hepatocarcinoma cells. However, the roles of caveolin-1 in cell-ECM adhesion and the mechanisms involved remain unknown. This study showed that caveolin-1 promoted the expression of the key α2,6-sialyltransferase ST6Gal-I and fibroactin-mediated adhesion of mouse hepatocarcinoma cell. Caveolin-1 up-regulated cell surface α2,6-linked sialic acid via stimulating ST6Gal-I transcription. Caveolin-1 might regulate ST6Gal-I expression through caveolin-1 scaffolding domain. Cell surface α2,6-sialylation was required for integrin α5β1-dependent cell adhesion to fibronectin, and an increase in α2,6-linked sialic acid on α5-subunit facilitated fibroactin-mediated focal adhesion kinase phosphorylations, suggesting that α2,6-sialylated α5-subunit promoted integrin α5β1-dependent cell adhesion. Taken together, these results demonstrate for the first time that caveolin-1 can up-regulate ST6Gal-I expression and further contribute to promoting mouse hepatocarcinoma cell adhesion to fibronectin by activating FAK-mediated adhesion signaling. This work was supported by grants from the Major State Basic Research Development program of China (2012CB822103), and National Natural Science Foundation of China (31000372, 31170774 and 31000618).

(212) Glycopharmacology: Changes in Tumor Tissue Glycan Expression in Response to Chemotherapy

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A workflow to evaluate the effects of cancer chemotherapies on the protein and glycan expression of membrane glycoproteins on the plasma membrane has been developed. This includes the use of metabolic labeling of glycoproteins with azide-sugar analogs in cell lines, plus and minus drug, and MALDI imaging and glycopeptide analysis of related tumor xenograft tissues, plus and minus drug. Two ceramide/ sphingolipid biosynthesis pathway inhibitors were tested. One is LCL-124 (from SphingoGene, Inc.), a mitochondrial targeted cationic ceramide derivative that initiates apoptosis via mitochondrial depolarization. The other is a sphingosine kinase-2 (SK2) selective inhibitor, ABC294640, currently in phase I/ IIa clinical testing for treating patients with advanced solid tumors. These compounds have been tested in prostate, kidney and pancreatic tumor cell lines, and in corresponding xenograft models in nude mice. Using high resolution FT-ICR MS imaging, the localization of each drug in the tumor xenograft tissues was determined. For N-glycan analysis, tumor slices were fixed in ethanol washes, sprayed with recombinant PNGaseF (20 mU) and incubated for 2 hrs. Tissues were sprayed with DHB matrix and processed for MALDI FT-ICR MS imaging. For each drug, multiple glycan species were differentially expressed in the drug treated or non-treated tumor tissues. The spatial localization of drug metabolite was also compared to the released glycan expression patterns. Also, differential molecular imaging of glycolipid species (GluCer, LacCer) in these same tissues was done. For cell culture analysis, prostate and pancreatic tumor cell lines, plus and minus drug. Two ceramide/ sphingolipid species (GluCer, LacCer) in these same tissues was done. For cell culture analysis, prostate and pancreatic tumor cell lines, plus and minus the ABC294640 inhibitor were co-cultured with ManNacAz or GalNAcAz sugars for incorporation. Following alkyne-bead capture of the sugar azide-glycoproteins, LC-MS/ MS was done to identify differential expression of glycoproteins, plus and minus the ABC294640 drug. Many changes in glycoprotein expression were identified. For example with both sugar analogs, drug treatment resulted in increased incorporation into integrin beta-2, integrin alpha-2, and ICAM1, with corresponding decreases in LAMP2 and integrin-betaV. Glycopeptide analysis with the same cell lines and tumor tissues is ongoing to link drug-induced changes...
in glycan composition and glycoprotein expression to specific glycoprotein species.

(213) Development of an ELISA to measure SULF2 levels in human blood
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As extracellular enzymes that are both tethered to the cell membrane and secreted, the SULFs and their heparan sulfate proteoglycan substrates are present in the extracellular environment. We hypothesize that the blood levels of SULFs or their substrates may serve as biomarkers for the early detection of NSCLC, malignant astrocytoma and other cancers. To test this hypothesis we have developed an ELISA for the detection of SULF2 in human blood. After testing a number of different strategies including using different combinations of our anti-SULF2 mAbs, we determined that a sandwich ELISA with capture mAb 5C12 followed by detection with biotinylated mAb 8G1 was best for the most sensitive detection of SULF2. By running a mouse IgG control in parallel with our 5C12 capture we determined that a significant subset of human blood samples contained human anti-mouse antibodies (HAMA). By adding 25 µg/mL of normal mlgG to each sample we were able to block HAMA in most cases. To demonstrate the suitability of the ELISA for use with plasma/serum, we performed spike and recovery assays using conditioned media from a breast cancer cell line (MCF7) containing known amounts of SULF2 added to plasma or serum. At low SULF2 concentrations (8.3 pg/well) added to 50 µl of serum, the mean % spike recovery was slightly over 100% and at high SULF2 concentrations (75 pg/well) the mean % spike recovery was ~80%. Similar results across three different laboratories demonstrate the assay robustness. Using the assay, we found that normal human serum and plasma contain a mean level of 155 and 60 pg/ml of SULF2, respectively. We have performed preliminary analyses of SULF2 levels in blood from cancer patients. We found that mean SULF2 levels in blood are significantly elevated in patients with NSCLC and malignant astrocytoma as compared to healthy blood donors. Additional studies in larger patient cohorts are needed to substantiate these findings.

(214) Analysis of serum fucosylated haptoglobin in chronic liver diseases as a potential biomarker of hepatocellular carcinoma development
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Objectives: Fucosylation is one of the most important glycosylation events involved in cancer and inflammation. We developed a lectin antibody enzyme-linked immunosorbent assay (ELISA) kit to measure fucosylated haptoglobin (Fuc-Hpt) which was identified as a novel biomarker of cancer. We investigated Fuc-Hpt as a marker in chronic liver diseases, especially hepatocellular carcinoma (HCC). Methods: We measured serum Fuc-Hpt levels by lectin antibody ELISA in 318 patients with chronic liver diseases, including 81 patients with liver cirrhosis (LC), 92 patients with HCC, and 145 patients with chronic hepatitis (CH). Serum Fuc-Hpt levels were measured at three different time points during a long-term follow-up period of 7 years (1996-2003) in 19 HCC patients who had LC. Serum Fuc-Hpt levels were also examined with short-term follow up (2009-2012) in 16 HCC patients who had LC. Results: Serum Fuc-Hpt levels increased with progression of liver disease. Patients with LC and HCC showed significantly increased Fuc-Hpt levels in comparison to patients with CH or healthy volunteers. Although Fuc-Hpt levels were slightly higher in HCC patients than in LC patients, the difference was not statistically significant. Fuc-Hpt levels correlated with hepatitis activity in CH patients. PIVKA-II and Fuc-Hpt were more useful than AFP and AFP-L3 for detecting HCC by computed tomography or ultrasound in patients with LC who had long-term follow up. More than 80% of LC patients with long-term follow up showed increased Fuc-Hpt during hepatocarcinogenesis. Approximately 38% of patients with LC and early-stage HCC who had short-term follow up showed a gradual increase in Fuc-Hpt before imaging diagnosis. Conclusions & Discussions: These results suggest that Fuc-Hpt is a novel and potentially useful biomarker for predicting progression of liver diseases and development of HCC. Further studies about possible applications of Fuc-Hpt to human liver diseases will be discussed.

(215) The Human Platelet Glycome and Impact of Storage
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Platelets have the shortest shelf-life of all major blood components, a factor that significantly complicates platelet transfusion. Changes to surface glycans during platelet storage has been demonstrated using lectins and related biochemical approaches. We have taken an effort to further understand these issues in a structural context that includes a study of the N- and O-linked glycans and glycosphingolipids (GSLs) using HPLC, LC-MS/MS, and sequential mass spectrometry (MS) approaches. Human platelets were obtained from commercial blood bags, and from healthy volunteers. Platelets were isolated by standard methods, then lyophilized and sonicated with chloroform/methanol to extract GSLs. After treating with trypsin/chymotrypsin the N-glycans were enzymatically released from the glycopeptides and isolated. In a subsequent step, the O-glycans were released from the remaining glycopeptides by classical reductive β-elimination. The N-glycan fractions were labeled with 2-AA for quantification by HPLC, or alternatively, permethylated for MALDI-TOF-MS and nanospray (NSI) MS disassembly. Quantification of permethylated O-glycans was performed by...
We have established a rapid and highly efficient method for MALDI-TOF-MS and NSI-MS Selected sialofucosyl-moieties observed on N-glycan antennae were detailed by MS and library spectral-matching. Fresh platelets included a significant amount of high-mannose (Man9-Mann) and asialo complex glycans, however, the N-glycans are dominated by a diverse range of complex sialylated structures with two to four antennae, up to four NeuAc residues, antennary fucosylation, and five or more lactosamine extensions. The O-linked fractions comprise core-1 and core-2 glycans having zero, one, or two NeuAc A significant decrease in sialylation was confirmed during conventional room temperature storage. Quantitative analysis of the more structurally complex N-glycan pools and analysis of the GSL structures is ongoing.

(216) Glycomics of resting and activated human platelets
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Cardiovascular diseases are the most common causes of death in developed countries and their prevalence rate is rapidly growing in developing countries. Genome-wide association studies (GWAS) have identified associations between ABO blood group status and HLBS (heart, lung, blood, and sleep) disorders. ABO glycans are expressed on glycoproteins and glycosphingolipids (GSLs) of cells and tissues relevant to HLBS disorders, including endothelial cells and platelets. Several major HLBS disorders, particularly acute myocardial infarction and acute lung injury, are characterized by endothelium dysfunction and platelet-driven thrombosis. Therefore, a comprehensive understanding of the disposition of ABO glycan expression by endothelial cells and platelets is essential for elucidating the mechanisms by which these glycans influence risk for cardiovascular disease. Toward this end, we have undertaken the structural characterization of GSL, and N-and O-linked glycoprotein glycans in resting and activated platelets. We have established a rapid and highly efficient method for isolating human circulating platelets from whole blood with efficient magnetic bead-depletion of contaminating leukocytes and red blood cells and inhibition of platelet activation during isolation in order to collect resting platelets. Resting platelets were maximally activated by incubation with 10 uM phorbol 12-myristate 13-acetate (PMA) at 37°C. For glycomic analyses, we have optimized an extraction method for harvesting GSLs and glycoproteins from minimal amounts of human platelets. The resulting preparations of GSLs were permethylated and analyzed by direct infusion into an NSI-LTQ/Orbitrap mass spectrometer. N-linked glycans are enzymatically released and O-linked glycans are released by reductive beta-elimination from glycoprotein preparations prior to permethylation and subsequent quantitative analysis by mass spectrometry. For GSLs, expression levels of lactosylceramide (LacCer) and of the sialylated form of LacCer, ganglioside GM3, were significantly decreased and increased, respectively, upon activation. For N-linked glycans, disialylated, core-fucosylated, biantennary N-glycans were increased in activated platelets compared to resting platelets. These results are consistent with a systemic upregulation in human platelet glycoconjugate sialylation during activation.

(217) Variations in Platelet Surface Glycans Among Healthy Volunteers
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Platelets have the shortest shelf-life of all major blood components and are the most difficult to store complicating platelet transfusion practices. Transfused fresh radiolabeled autologous platelets differ significantly in recovery and survival among healthy subjects, however the cause of the inter-individual differences remains unclear. We demonstrated that the loss of sialic acid from the surfaces of cold-stored and transfused platelets promotes their clearance by Ashwell Morell receptors. The loss of platelet surface sialic acid correlates with increases in surface sialidase activity during platelet storage. Here we investigated whether fresh platelets from individual donors exhibit differences in surface sialidase expression and glycan exposure, which may affect post-transfusion platelet recovery and survival. Platelets were isolated by standard methods from the venous blood of healthy volunteers and analyzed by flow cytometry for surface β-galactose using FITC-conjugated E. cristagalli lectin (ECL). Platelet surface sialidase expression was measured by flow cytometry using antibodies to sialidases Neu1 and Neu3. Sialidase activities were assayed using standard methods. Platelet uptake by hepatocytes was measured by using the human hepatoma cell line HepG2. We found that terminal galactose on freshly-isolated platelet glycoproteins varies considerably among healthy subjects: Seven of ten individuals had low levels of exposed galactose (15.3 ± 4.1, MFI) and three subjects exhibited significantly higher levels of terminal galactose as detected by flow cytometry using lectins. These results were confirmed by repeated measurements of the same individuals at three different time points. Reduced sialic acid content correlated with increased surface sialidase activity and expression. Platelets with high terminal galactose were ingested with a higher rate by HepG2 cells, i.e via Ashwell Morell receptors. Importantly, individuals with low sialic acid levels correlate with low platelet counts at steady state. Conclusion: Our results show that fresh platelets from healthy individuals vary in surface sialidase activity and sialic acid content. Collectively we propose that individual platelet counts are regulated by surface sialic acid content and that the surface sialic acid could represent a factor that affects the recovery and survival of transfused fresh platelets.

(218) Abberant Type-2-lactosaminoglycan synthesis severely impaires thrombopoiesis
Silvia Giannini, Antonija Jurak Begonja, Max Adelmann, Karin Hoffmeister
BWH and HMS
Platelet recovery is crucial to avoid bleeding complications following myelosuppressive and myeloablative chemotherapy. Platelets are produced by megakaryocytes (MKs), which develop and mature from hematopoietic stem cells (HSC) in the bone marrow. Mature MKs interact with sinusoidal bone marrow endothelial cells to form transendothelial pseudopods, called proplatelets, from which platelets are released into the bloodstream. We have demonstrated that platelet survival in circulation is dependent on correct glycan expression. Here, we investigate the role of Type-2-Lactosaminylglycans (Type-2-LacNAc) in platelet production. β1,4Galactosyltransferase 1 (β4GalT1) is a major enzyme involved in Type-2-LacNAc synthesis which adds 1,4-Galactose (Gal) to terminal N-Acetylglucosamine (GlcNAc) to form β1,4Gal-GlcNAc (Type-2-LacNAc). We here show that the majority of β4GalT1−/− mice die in utero between E15.5 and E16.5. Few β4GalT1+ mice survive until adulthood, exhibiting severe macro-thrombocytopenia despite normal platelet clearance. To obtain platelets lacking functional β4GalT1, bone marrow chimeras were produced by transplanting β4GalT1−/− fetal liver hematopoietic stem cells (FLHSC) into lethally irradiated wild type mice. We were unable to detect circulating β4GalT1+ platelets despite existing mature bone marrow MKs. In contrast we measured normal circulating β4GalT1−/− white blood cells, showing that β4GalT1−/− FLHSC can only partially restore bone marrow hematopoiesis. Moreover, β4GalT1 deficient fetal liver MKs differentiate and mature normally, as judged by number, morphology, ploidy and expression of main surface glycoprotein’s, but have severely impaired proplatelet production in vitro. Taken together, our data strongly support the notion that glycosylation mediated by β4GalT1 is crucial for platelet generation, but not for maturation and differentiation of MKs in vitro and in vivo. We demonstrate here a novel role for post-translational glycan modification in platelet genesis.

(219) Regulating hematopoietic equilibrium by manipulating circulatory ST6Gal-1
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Regulating blood cell production is crucial to meet demands in combating invading pathogens and in maintaining hematopoietic equilibrium. Insufficiency of hematopoiesis, from cancer therapy, exposure to toxins, or from unknown etiology, poses significant risk of mortality. Elucidation of the factors regulating the bone marrow microenvironment remains the principal obstacle in mechanistic understanding and development of novel therapeutic strategies. Our lab has recently redefined the traditional paradigm of "intrinsic" or "cell-internal" glycosylation by describing a new "extrinsic" mechanism, where outside sources of glycosyltransferases modify existing cell surface glycans and alter their interactions with the extracellular environment. The specific glycosyltransferase, ST6Gal-1, present in the blood can delay hematopoietic stem/progenitor cell (HSPC) proliferation, presumably by conveying an α2,6-sialic acid linkage onto HSPC cell surfaces. To investigate this process in vivo, we developed a genetically modified an subcutaneously implantable B16-F10 to elevate circulatory α2,6-sialyltransferase activity (~40-fold over background in vitro and >10 fold in wild-type mice) C7BL/6 mouse model. The B16St6gal1 WT mice had a >50 percent reduction in overall bone marrow granulocyte and B-cell populations, as well as a 2-fold increase in the number of lineage negative (lin-) cells, compared to no-tumor and unmodified tumor controls. The B16 mice also show a 1.8 fold increase in the number of myeloid colony forming units (CFU) from total bone marrow as compared to the controls. These observations are consistent with circulatory ST6Gal-1 delaying hematopoietic development into end-effector cells, and consequently inflating hematopoietic progenitor pools immediately preceding the blockade. Upon subcutaneous implantation of the B16 tumors into St6gal1-KO mice, SNA reactivity can be detected in the lin-cells of the bone marrow and spleens, demonstrating blood-borne ST6Gal-1 can sialylate cells in a SNA-unreactive, ST6Gal-1 deficient animal. Taken together, we proposed the idea that the extrinsic mechanism of ST6Gal-1 sialylation can be leveraged to modify hematopoietic parameters for clinical benefit. Up-regulation of ST6Gal-1 can drive HSPC quiescence when control of myelo-proliferative disorders is desired. Conversely depletion of circulatory ST6Gal-1 may aid recovery from myelo-suppressive states. (Funded by NIH Program of Excellence in Glycosciences award P01HL107146 and NIH R01AI56082)

(220) Inducing Host Protection in Sepsis by Rapid Activation of the Ashwell-Morell Receptor
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Sepsis is a systemic inflammatory response in the presence of a pathogenic organism in the bloodstream. Once in the bloodstream, pathogens often induce a hyperactive inflammatory cascade that can progress to disseminated intravascular coagulation with severe thrombosis, organ failure, and host death. With current limited understanding of pathogen-host interactions, sepsis remains a debilitating and deadly syndrome with few treatment options. We have previously reported that the endocytic Ashwell-Morell Receptor (AMR) of hepatocytes detects pathogen remodeling of host glycoproteins by neuraminidase in the bloodstream and mitigates the lethal coagulopathy of sepsis. We have now further investigated the mechanism of host protection by the AMR during the onset of sepsis in response to the de-sialylation of blood glycoproteins by the NanA neuraminidase of the ubiquitous lethal pathogen- Streptococcus pneumoniae (SPN). We find that the AMR selects among potential glycoprotein ligands unmasked by neuraminidase...
activity to eliminate from blood circulation those that primarily contribute to coagulation and thrombosis. This protection is attributable in large part to the rapid induction of a moderate thrombocytopenia by the AMR. We further show that neuraminidase activity in the blood can be manipulated to induce the clearance of AMR ligands including platelets thereby pre-activating a protective host response in early sepsis that further lessens disseminated intravascular coagulation and promotes survival. The regulation of circulating platelet and coagulation factors by the AMR represents a posttranslational process that is imperceptible to multiple studies of genomic variation and transcriptional outputs in sepsis. This SPN pathogen-host interaction is likely to have been ongoing through millions of years of evolution providing a selective pressure favoring the endocytic AMR response to counterpart perturbation of the host coagulation system.

Glycosphingolipids stabilize E-selectin mediated slow rolling of human leukocytes on endothelial cells

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E-, P-and L-selectin constitute a family of C-type lectins that mediate the first step of a cell adhesion cascade that eventually recruits leukocytes to sites of inflammation. Among the leukocyte cell-surface glycoconjugates, P-selectin glycoprotein ligand-1 (PSGL-1, CD162) is the most prominent ligand for L-and P-selectin in both human and mouse leukocytes. The precise ligands for E-selectin remain unidentified on human granulocytes, and additionally the glycosyltransferases mediating E-selectin ligand biosynthesis on human leukocytes may be distinct from that in mice (Buffone et al. 2013). To better characterize the human E-selectin ligands, we developed HL-60 cells stably transduced with shRNA (Buffone et al. 2013). To better characterize the human E-selectin ligands, we developed HL-60 cells stably transduced with shRNA (Buffone et al. 2013). To better characterize the human E-selectin ligands, we developed HL-60 cells stably transduced with shRNA (Buffone et al. 2013). To better characterize the human E-selectin ligands, we developed HL-60 cells stably transduced with shRNA (Buffone et al. 2013).

The effect of gene silencing on the in-ligands, we developed HL-60 cells stably transduced with shRNA (Buffone et al. 2013).

HL-60 cell rolling, with instantaneous velocities as high as 300 µm/ sec, was also evident upon following individual cell trajectories. Treatment of wild-type and UGCG− HL-60 cells with pronase resulted in reduced cell adhesion and increased cell detachment for both cell types, with the effects being more pronounced for the UGCG− HL-60s. Both differentiated wild-type and UGCG− HL-60 cells transmigrated across HUVEC monolayers. Together, the data suggest a crucial role for human glycosphingolipids in stabilizing E-selectin mediated cell capture and slow rolling on endothelial cells.

References


We have developed a new mass spectrometry workflow using the intelligent fragmentation of O-glycans via consecutive reaction monitoring to improve O-glycomics analysis. Using the intensity of selected fragment ions and the use of glycan standards we are able to increase both sensitivity and accuracy of quantitation compared to total ion mapping strategies. Furthermore, by taking an intelligent fragmentation strategy we are able to identify and quantify changes in isobaric glycan structures between samples. The uniformity of this method of data collection also lends itself to automated data analysis that we have in early stages of development. The limit of detection and linearity of response for quantification of this method has been examined using serial dilutions of O-glycans recovered from a standard sample, mouse brains. To exhibit the clinical relevance, this method has been successfully applied to distinguish dystroglycanopathy samples, including both tissues from mouse models and blood samples from human patients, from control samples. Given that the current diagnosis for congenital muscular dystrophy, including the dystroglycanopathies, relies on an invasive muscle biopsy, the development of a diagnostic blood test paves the way for following efficacy in therapeutic trials aimed at improving O-mannosylation in a non-invasive and sensitive manner.

AAV-Mediated FKRP Gene Therapy to Rescue Functional Glycosylation of α-Dystroglycan in Dystrophic Mice

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Mutations in the fukutin related protein (FKRP) gene are commonly associated with a wide range of disease phenotypes that exhibit mild-to-severe characteristics (i.e., congenital muscular dystrophy, mental retardation, and cerebellar cysts). The main biochemical feature of these diseases is the abnormal glycosylation of α-dystroglycan (α-DG)-a protein that is critical for linking the...
dystrophin-glycoprotein complex to extracellular matrix proteins. Currently, there is a lack of effective treatment options available for these types of diseases. To investigate the potential of gene therapy, we examined the therapeutic effects of systemic FKRP gene delivery by adeno-associated virus serotype 9 (AAV9) into a dystrophic mouse model containing a knock-in p448L proline-to-leucine missense mutation (FKRP). This mouse strain presents moderate muscular dystrophy attributes, with early onset of skeletal muscle wasting, but lacks defects in the central nervous system. Mice at the age of 8-10 days were given a single dose of AAV9-FKRP via intraperitoneal injection and subsequently examined 11-12 months later. Our results show that systemic gene delivery resulted in long-term FKRP expression in all muscles types examined, with higher levels in cardiac muscle. Consequently, expression of AAV9-FKRP restored functional glycosylation of α-DG in the skeletal and cardiac muscles. Significant improvement in dystrophic pathology, muscle functions, and serum creatine kinase levels was also observed. Limited FKRP transgene expression was detected in kidney and liver with no observable cytotoxic effects. Our results suggest that AAV-mediated FKRP gene replacement therapy is a viable therapeutic strategy for FKRP-related dystroglycanopathies.

(224) Glycosaminoglycans Recovered from Alzheimer’s Disease Tissue Culture

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Glycosaminoglycans (GAGs) participate in the development of β-Amyloid neurofibrillary tangles characteristic of Alzheimer’s disease (AD). This work uses cell media of cultures grown previously (1) to collect and purify GAG. Samples were defatted over 48 hours with acetone, then digested with pronase and benzonase. Insoluble material was removed through centrifugation. The digest was further purified with DEAE-Sephrosepre-washed with 20 mM Tris-HCl, pH 7.5, 0.1M NaCl and eluted with 6 mL of 20 mM Tris-HCl pH 7.5, 2M NaCl. GAG were removed by beta-elimination in 0.7 mL 10% (w/v) NaBH in 2 N NaOH, incubated at 4 C overnight and combined with glacial acetic acid till the pH was neutral. The isolated GAG was desalted prior to treatment with enzyme. Enzymatic reactions were completed using Chondroitinase ABC or heparinase I, II and III. Produced disaccharides were separated using an Agilent Waters Spherisorb Analytical column HPLC (SAX-HPLC) with sodium phosphate gradient (2.5 mM, pH 3.5, to 1.2 M NaCl), and post-column detection with 2-cyanoacetate as fluorescent label. The data show heparan sulfate (HS) oligosaccharides as the most abundant products in the media of β-Amyloid Alzheimer’s disease; the controls had 37% (W/W) more HS. Chondroitin sulfates (CS) was the second GAG; its yields were about the same in AD and controls. HS is the major oligosaccharide of Alzheimer’s D vs. prenatal and neonatal rats used by other workers. A study of the disaccharide components of HS shows that Alzheimer’s D has a greatly reduced amount of highly sulfated disaccharides in comparison to that of the controls.

(225) Heparan sulfate proteoglycans mediate internalization and propagation of specific proteopathic seeds

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Tauopathies are a class of neurodegenerative disorders characterized by the pathological accumulation of microtubule-associated protein tau in the human brain. In the early stage of disease, tau pathology is often restricted to discrete and stereotyped regions of the brain. With disease progression, the pathological changes typically spread through the nervous system according to specific anatomical patterns. Emerging evidence demonstrates that tau aggregates are capable of transcellular spread whereby an aggregate formed in one cell is released freely into the extracellular space, enters a neighboring cell, and converts the natively folded tau protein into an aggregated, fibrillar form. Thus, misfolded tau aggregates capable of transcellular spread may directly serve as an agent of disease progression. Here, we describe the mechanism by which aggregated extracellular proteins such as tau and α-synuclein bind and enter cells to trigger intracellular fibril formation. Prior work indicates that prion protein aggregates bind heparan sulfate proteoglycans (HSPGs) on the cell surface to transmit pathologic processes. Here, we find that tau fibril uptake also occurs via HSPG binding. This is blocked in cultured cells and primary neurons by heparin, chlorate, heparinase, and genetic knockdown of a key HSPG synthetic enzyme, Ext1. Interference with tau binding to HSPGs prevents recombinant tau fibrils from inducing intracellular fibril formation. In vivo, a heparin mimetic, F6, blocks neuronal uptake of stereotactically injected tau fibrils. Finally, uptake and seeding by α-synuclein fibrils, but not huntingtin fibrils, occurs by the same mechanism as tau. This work suggests a unifying mechanism of cell uptake and propagation for tauopathy and synucleinopathy.

(226) Characterization and analysis of the 5D4-reactive keratan sulfate expressed in the central nervous system of SOD1G93A, an ALS model mouse

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The building blocks of keratan sulfate (KS) glycosaminoglycan are repeating disaccharides of Galβ1-4GlcNAc. Residues of Gal and GlcNAc can be modified with sulfate groups at their C-6 positions. The 5D4 monoclonal antibody recognizes sulfated KS oligosaccharides that consist of Gal(6S)β1-4GlcNAc(6S), which has been
extensively used to evaluate KS expression. KS is known to play a critical role in neuronal regeneration/sprouting after injury in the central nervous system (CNS). Amyotrophic lateral sclerosis (ALS) is a devastating motor neuron-degenerative disease. Recently, we reported that the 5D4-reactive KS is newly expressed in the spinal cord of SODG93A, an ALS model mouse (Hirano, PLoS ONE 2013). To characterize the 5D4-reactive KS and analyse expression of enzymes responsible for biosynthesis of KS in ALS pathogenesis, biochemical and immunohistochemical assays were performed. We have found that the 5D4-reactive KS molecules were modified with sialic acid and fucose and that the KS was susceptible to beta-elimination but not to PNGase F treatment. GlicNac6ST1 was up-regulated while KSGal6ST was not in SOD1G93A mice. Interestingly, the 5D4-reactive KS is also expressed in the brainstem of SOD1G93A mice. We corroborated that the 5D4 epitope is abolished in the spinal cord and brainstem of SOD1G93A/GlicNac6ST1 mice. Transfection studies revealed that GlicNac6ST1 and KSGal6ST cooperated in the expression of the 5D4 KS epitope in HECAa cells. These results indicated that GlicNac6ST1 is essential for biosynthesis of the 5D4-reactive KS in the CNS of SOD1G93A mice and that expression of 5D4 and GlicNac6ST1 may be also associated with degeneration of the upper motor neuron system in ALS pathogenesis.

(227) The function of N-glycans attached to the Fc domain of IgGs in the progression of amyotrophic lateral sclerosis (ALS)

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ALS is a fatal neurodegenerative disease caused by degeneration of upper and lower motor neurons. In a small scale study, we recently found a distinct glycan, bisecting bi-antennary without core fucose; A2BG2, in IgG derived from ALS-patient sera. The glycan increased IgG affinity to CD16 (FcγRIIIA) on effector cells and consequently stimulated effector cell activity and lysis of motor neuron cell lines, in vitro. In situ study revealed over-expression of CD16 and co-localization of intact ALS-IgGs with CD16 and with activated microglia cells of ALS transgenic mice; mSOD1. Based on these results, we studied the roles of Fc-glycans in ALS progression using mSOD1 mice. Analyses of the Fc-glycans enriched from mSOD1 and littermate blood at four different clinical severities; pre-symptomatic, onset of symptomatic, progressive disease with severe weakness and prior to paralysis and at the end-stage of the disease revealed a linear correlation between the Fc-glycans and the clinical severity. The data were confirmed by quantitative PCR of glycosyl- trasferase mRNA extracted from plasma B-cells and by in vitro antibody-dependent cell-cytotoxicity. We next injected CD16 competitive inhibitor into the cerebrospinal fluid of symptomatic mSOD1 mice and followed mouse weight, disease progression and severity. Data showed low rate of disease progression and score in mSOD1-treated mice relative to mSOD1 mice administered with placebo. We are now modulating the IgG interactions with its receptors on effector cells by fabricated-Fc with standardized N-glycans. The establishment of structure-activity profiles should lead to understand the involvement of IgG-N-glycans in the ALS progression and to prolonging patient’s lives.

(228) A congenital disorder of deglycosylation: Biochemical characterization of N-glycanase 1 deficiency in patient fibroblasts

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N-glycanase 1, encoded by NGLY1, catalyzes the deglycosylation of misfolded N-glycosylated proteins. The glycoamidase also called PNGase is highly conserved across species. Using whole-genome and -exome sequencing, we recently identified three cases with mutations in NGLY1. The patients all show developmental delay, seizures, involuntary movements, peripheral neuropathy, abnormal liver function, and absent tears. The nonsense and frame shift mutations in NGLY1 for these three cases resulted in drastic reduction of N-glycanase 1 protein in patient-derived fibroblasts. Since N-glycanase 1 generates free oligosaccharides (fOS) from misfolded glycoproteins, we probed enzymatic activity indirectly by measuring the presence of [2-H] mannose-labeled fOS following short-term labeling. N-glycanase 1 deficient fibroblasts produced 2-3 fold less free glycans than controls and showed an altered size distribution. Applying a recently established cellular deglycosylation dependent Venus (ddVenus) fluorescence assay, we found that patient fibroblasts had dramatically reduced fluorescence, which was completely restored by complementation with wild type N-glycanase 1 cDNA, but not by mutants lacking enzymatic activity. Since deglycosylation of misfolded glycoproteins precedes proteasomal degradation, we speculated that N-glycanase 1 deficiency might also accumulate misfolded glycoproteins, leading to ER stress. To test this hypothesis, we firstly transformed patient cells with vectors expressing ERAD substrates, TCRα-GFP and A1AT-NHK-GFP. We observed 5-10 fold increase of GFP fluorescence and accumulation of those misfolded proteins in the cytoplasm. However, under physiological conditions, we did not observe abnormalities of the three branches of UPR signal transduction (IRE1, PERK, and ATF6) in patient fibroblasts, indicating that the N-glycanase 1 deficiency itself is not sufficient to cause ER stress. We also explored other potentially relevant pathways, like proteostasis (HSP40, HSP70 and HSP90), ubiquitination (ubiquitin and FBXO-6) and autophagy (LC3 and phospho-eEF2). The preliminary experiments showed no alterations in any of these. Taken together, mutations in NGLY1 cause the first "Congenital Disorder of Deglycosylation". Our biochemical results showed that the mutations reduce N-glycanase 1 protein level and enzymatic activity and cause cytoplasmic accumulation of misfolded
glycoproteins. The mechanism by which N-glycanase I deficiency causes the clinical phenotypes is under investigation. The work is supported by the Bertrand Might Research Fund.

(229) TMEM165 deficiency is associated with cartilage and glycosylation defects in zebrafish
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Congenital Disorders of Glycosylation (CDG) are a group of heterogenous inherited diseases characterized by deficient glycosylation. The challenge is to identify new genetic causes for CDG and to study the pathophysiology of the different types of this disease. For the affected individuals described in this study, the N-glycan analysis revealed a relative increase in the undersialylated and underlactosylated glycans. However, this did not allow us to pinpoint the underlying molecular defect. But through a combination of autozygos-ity mapping and expression analysis in two affected siblings with an abnormal serum-transferrin isoelectric focusing test (type 2), we identified \textit{TMEM165} as a novel gene involved in CDG-II. These patients present with psychomotor retardation and an unusual skeletal phenotype with growth deficiency and joint anomalies. \textit{TMEM165} encodes a putative transmembrane protein of 324 amino acids whose cellular functions are unknown. To assess the phenotypic consequences of \textit{Tmem165} loss of function during the early development of zebrafish, two morpholinos (MO) were tested: a translation blocking MO and a splicing blocking MO. In general, knockdown of \textit{Tmem165} in zebrafish embryos result in a shorter body length and misshapen head. \textit{TMEM165} is widely expressed in zebrafish, resulting in a very broad and diffuse staining pattern. A single orthologue and a single homologue with 79\% identity on protein level was found, suggesting that it is functionally conserved. Motivated by the fact that \textit{TMEM165}-CDG patients exhibit growth retardation and skeletal malformations, we were prompted to analyze developing cartilage in \textit{tmem165} morphants in detail by staining with Alcian blue, a dye that binds acidic components of the extracellular matrix. \textit{Tmem165} morphant embryos displayed several defects in cartilage morphogenesis, including an abnormal Meckel’s cartilages, misshapen palatoquadrate and ceratohyal structures. The pharyngeal cartilage elements were shown to be shorter than those of control larvae. Coinjection of \textit{tmem165} mRNA significantly improved the size and shape of the craniofacial cartilages. Interestingly, the expression of chondroitin sulfate in the \textit{tmem165} morphants was reduced in the ceratohyal and Meckel’s cartilage. Thus, we showed that a zebrafish morphant have cartilage and glycosylation defects and thus is a suitable model for the study of the \textit{TMEM165} deficiency in patients.

(230) Surface coupling of P-selectin glycoprotein ligand-1 onto mesenchymal stem cells enables leukocyte-like cell tethering and rolling
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Mesenchymal stem/ stromal cells (MSCs) are an important candidate for cell-based therapy since they can be easily isolated and expanded, secrete beneficial paracrine factors, and differentiate into multiple lineages. Since the endothelium at sites of injury and inflammation often express adhesion molecules belonging to the selectin family, methods to endow MSCs with selectin-ligands can enhance the efficacy of cell delivery and tissue engraftment. Here, we describe a construct 19Fc[FUT7], where the first 19 amino acids of the pan-selectin ligand PSGL-1 (P-selectin glycoprotein ligand-1) was fused to a human IgG tail. When expressed in HEK293T cells over-expressing the α(1,3)fucosyltransferase FUT7, 19Fc[FUT7] is decorated by a core-2 sialyl-Lewis-X sialofucosylated O-glycan. The non-covalent coupling of this protein onto MSC surface using palmitated protein G (PPG) enhanced cell binding to E- and P-selectin under hydrodynamic shear, without altering MSC\textsubscript{1,2} multipotency. MSC’s functionalized with 19Fc[FUT7] were captured/ tethered onto stimulated endothelial cell monolayers at wall shear stresses up to 4 dyn/cm\(^2\). Once captured, the cells rolled robustly up to the highest shear stress tested, 10 dyn/cm\(^2\). Unlike previous work where MSCs were captured onto selectin-bearing substrates at low or no-flow conditions, the current work presents a ‘glycan engineering’ strategy to enable leukocyte-like capture and rolling.

(231) From SIGLEC14 polymorphism toward the systemic biomarkers of exacerbation of chronic obstructive pulmonary disease (COPD)
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Rationale: Chronic obstructive pulmonary disease (COPD) is a leading cause of mortality worldwide. Exacerbation, or episodic worsening of symptoms, often results in hospitalization and even mortality of COPD patient. The diagnosis of exacerbation relies on the clinical manifestation of the patient, and is inevitably subjective. Quantifiable biomarker of COPD exacerbation has been sought after, but it remains elusive. We have previously demonstrated that the COPD patients who lack Siglec-14, a myeloid cell lectin that
recognize sialylated bacteria *Haemophilus influenzae* and trigger cell activation, are less prone to exacerbation. We hypothesized that the soluble mediators secreted by myeloid cells in response to Siglec-14 engagement are involved in the self-propagating inflammatory cycle leading to exacerbation, and that these mediators may be utilized as systemic biomarkers of exacerbation. Methods: Gene expression levels in Siglec-14(+) versus Siglec-5(+) myeloid cell lines stimulated with *Haemophilus influenzae* were performed to measure the involvement of various signaling pathways in these studies. Subsequently, mechanistic studies were performed to evaluate the involvement of various signaling transduction pathways. Our in vitro studies using H9C2 cells reveal that LacCer (100 µM) can induce hypertrophy and raise the levels of BNP/ANP mRNA. However, comparative study using 100 µM concentration of other GSL’s e.g. Cer, GlcCer, Globose3Cer, Globose4Cer, Gangliosides (e.g. GM3, GM1, GD3, GD1a, GD1b), sulfatides, sphingosine and ceramide did not induce hypertrophy. The exposure of H9C2 and primary cultured neonatal rat cardiomyocytes to LacCer, dose-dependently activated hypertrophic parameters. Maximum increase in cardiac hypertrophy (3 fold compared to control) was observed using 100 µM of LacCer. This phenotype was observed as time dependent and LacCer showed increase in cell surface area, cell volume, protein content and increased expression of fetal genes. LacCer induced hypertrophy is mediated by oxidative stress and LacCer was found to be associated with altered gene expression as well. LacCer also activated MAP kinases implicated in signaling pathways of cardiac hypertrophy. Conclusively, we report here for the first time that LacCer induces hypertrophy in cardiomyocytes. This work was supported by NIH grants PO-1-HL-107153-01.

**(232) Lactosylceramide induces hypertrophy in cardiomyocytes**

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Glycosphingolipids (GSLs) are integral component of the surface of essentially all types of cells and tissues including heart. Lactosylceramide (LacCer), a member of the neutral GSL family is synthesized from Glucosylceramide (GlcCer) by Lactosylceramide synthase, plays a pivotal role in the biosynthesis of complex GSLs and imparts several critical phenotypes. The role of GSLs in cardiac system is yet unknown and physiological and pathophysiological consequences of myocardial sphingolipid species and their regional differences in myocardium remain obscure. We have determined the efficacy of wide variety of GSL’s to affect cardiac hypertrophy using cultured rat cardiomyocytes e.g. H9C2 cells and primary neonatal rat cardiomyocytes. We employed multiple criteria to assess hypertrophy in our studies. These were: a) measurement of [3H]Leucine incorporation into protein, b) measurement of cell size and morphology by immunofluorescence microscopy and c) Real time quantitative mRNA expression assay for atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). For comparison, we have also used phenylephrine (PE); a well-established agonist of cardiac hypertrophy in these studies. Subsequently, mechanistic studies were performed to measure the involvement of various signaling transduction pathways. Our in vitro studies using H9C2 cells reveal that LacCer (100 µM) can induce hypertrophy and raise the levels of BNP/ANP mRNA. However, comparative study using 100 µM concentration of other GSL’s e.g. Cer, GlcCer, Globose3Cer, Globose4Cer, Gangliosides (e.g. GM3, GM1, GD3, GD1a, GD1b), sulfatides, sphingosine and ceramide did not induce hypertrophy. The exposure of H9C2 and primary cultured neonatal rat cardiomyocytes to LacCer, dose-dependently activated hypertrophic parameters. Maximum increase in cardiac hypertrophy (3 fold compared to control) was observed using 100 µM of LacCer. This phenotype was observed as time dependent and LacCer showed increase in cell surface area, cell volume, protein content and increased expression of fetal genes. LacCer induced hypertrophy is mediated by oxidative stress and LacCer was found to be associated with altered gene expression as well. LacCer also activated MAP kinases implicated in signaling pathways of cardiac hypertrophy. Conclusively, we report here for the first time that LacCer induces hypertrophy in cardiomyocytes. This work was supported by NIH grants PO-1-HL-107153-01.

**(233) Inhibiting Glycosphingolipid glycosyltransferase activity Prevents Cardiac hypertrophy in apoE-/-mice fed western diet and C57 Bl-6 mice subject to trans-aortic constriction**

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One in three person world-wide suffers from hypertension and cardiac hypertrophy is one of the major contributing factors for this disorder. Whether glycosphingolipids and glycosyltransferases play a role in cardiac hypertrophy is not known. We have observed extensive cardiac hypertrophy in Apo-E-/-mice fed with high cholesterol and high fat diet(HCHF). This was accompanied by a marked increase in the activity of glucosylceramide synths and lactosylceramide synthase. The level of glucosylceramide and lactosylceramide in the left ventricular tissue and liver were increased. When mice were fed (by oral gavage) an inhibitor of glycosphingolipid glycosyltransferase; D-threo-1-phenyl -2-decanoylamino-3-morpholino-1-propanol-HCl(D-PDMP), daily for 6 months, it dose-dependently decreased the activity of these glycosyltransferases, and glycosphingolipid mass, the latter measured by tandem mass spectrometry. Most importantly, we noted amelioration of cardiac hypertrophy measured by ultra sound, left ventricular mass measurements, and protein/gene expression of atrial natriuretic peptide, BNP, and transforming growth factor (TGF-β). Trans-aortic constriction (TAC) in normal C57Bl-6 mice also causes extensive cardiac hypertrophy due to an increase in high blood volume and pressure. When these mice were fed D-PDMP at least 24 hr before TAC surgery, and daily up to six additional days, it completely reversed cardiac hypertrophy. In sum, inhibiting glycosphingolipid glycosyltransferase activity is a novel approach to mitigate cardiac hypertrophy in experimental mouse models. This work was supported by NIH grants PO-1-HL-107153-01.