Consortium for Functional Glycomics
Participating Investigator Meeting, May 17-18, 2005

Location: Balcony B, Natcher Conference Center – NIH
45 Center Drive, Bethesda, MD (see attached map)
Picture ID is required to enter building

AGENDA

Monday, May 16th
7:30 to 9:00 p.m. Open reception at Marriott Bethesda Hotel
5151 Pooks Hill Road at the Congressional Ballroom - Salon 1&2

Tuesday, May 17th (DAY ONE)
7:30 – 8:00 BREAKFAST

8:00 – 10:00 INTRODUCTION/CORE PRESENTATIONS

8:00 – 8:15 James Paulson (The Scripps Research Institute)
Overview of CFG (click here to view presentation)

8:15 – 8:30 Stuart Haslam (Imperial College London)
Overview of Analytical Glycotechnology Core (C) (click here to view presentation)

8:30 – 8:45 Steve Head (The Scripps Research Institute)
Overview of Gene Microarray Core (E) (click here to view presentation)

8:45 – 9:00 Peter Sobieszczuk (The Scripps Research Institute)
Overview of Mouse Transgenics Core (F) (click here to view presentation)

9:00 – 9:15 Sally Orr (University of California, San Diego)
Overview of Mouse Phenotype Core (G) (click here to view presentation)

9:15 – 9:30 Ola Blixt (The Scripps Research Institute)
Overview of Carbohydrate Synthesis/Protein Expression Core (D) (click here to view presentation)

9:30 – 9:45 Rick Alvarez (University of Oklahoma Health Sciences Center)
Overview of Protein-Carbohydrate Interaction Core (H) (click here to view presentation)

9:45 – 10:00 Questions for the Core Directors

10:00 – 10:30 MORNING BREAK

10:30 – 11:40 GALECTINS

10:30 – 10:50 Richard Cummings (University of Oklahoma Health Sciences Center)
Human galectin structures and activities: carbohydrate recognition and cell signaling

10:50 – 11:10 Hakon Leffler (Lund University)
Galectin phylogeny, specificity, and development of new inhibitors

11:10 – 11:25 Margaret Huflejt (Sidney Kimmel Cancer Center)
Diagnostic and drug discovery applications of printed glycan array in experimental and clinical oncology

11:25 – 11:40 Kate Rittenhouse-Olson (University of Buffalo)
Metastatic variants of a breast tumor line compared using the glyco-chip
11:40 – 1:10  LUNCH BREAK (lunch to be provided)
POSTERS at the Atrium area
DATABASE DEMONSTRATION in room 2As.10 conference room

1:10 – 1:50  TRANSGENIC MICE

1:10 – 1:30  Jamey Marth (University of California, San Diego)
Decoding glycan function by combining genetic and phenotypic approaches

1:30 – 1:45  Hermann Ziltener (University of British Columbia)
Homing of adult thymic progenitors is mediated by P-selectin and its ligand PSGL-1

1:45 – 3:25  FRONTIERS IN CARBOHYDRATE CHEMISTRY

1:45 – 2:05  Chi-Huey Wong (The Scripps Research Institute)
Programmable synthesis for glycobiology and medicine

2:05 – 2:25  Peter Seeberger (ETH Hönggerberg)
Chemical Glycomics: Automated Synthesis Enables Carbohydrate Arrays, Tools and Vaccines

2:25 – 2:45  Nicolai Bovin (Shemyakin Institute of Bioorganic Chemistry RAS)
Microscale spacer-arming and new approaches to surface “carbohydrating”

2:45 – 3:05  Peng George Wang (The Ohio State University)
Development of Glycopharmaceuticals: from small sugar containing molecules to carbohydrate polymer

3:05 – 3:25  Lai-Xi Wang (University of Maryland Biotechnology Institute)
Synthesis and functional study of HIV-1 glycopeptides

3:25 – 3:55  AFTERNOON BREAK

3:55 – 4:35  SIGLECS

3:55 – 4:15  Bruce Bochner (The Johns Hopkins School of Medicine)
Siglec-8: an inhibitory receptor on human eosinophils, basophils and mast cells

4:15 – 4:35  Paul Crocker (University of Dundee)
Comparing glycan binding specificities of mouse and human siglecs

4:35 – 5:30  TCR

4:35 – 4:55  Ian Wilson (The Scripps Research Institute)
Structural basis of CD1 ligand recognition and specificity

4:55 – 5:15  Mitch Kronenberg (La Jolla Institute for Allergy and Immunology)
Innate-like immune responses of carbohydrate containing antigens by the T cell receptors of NKT cells

5:15 – 5:30  Dapeng Zhou (University of Chicago)
The identity of natural NKT ligand: Implications for therapy of cancer, auto-immune and allergic disease

6:30 p.m.  SC Meeting at the Marriott Hotel in the Annapolis/Chesapeake room
(Steering Committee & Advisory Committee members only)
Wednesday, May 18th (DAY TWO)

7:30 – 8:00  BREAKFAST

8:00 – 11:45  GLYCOMICS/BIOINFORMATICS

8:00 – 8:20  Rahul Raman  (Massachusetts Institute of Technology)
Bioinformatics and the construction of relational databases for functional glycomics

8:20 – 8:40  Anne Dell  (Imperial College London)
High throughput mouse and human glycomics

8:40 – 9:00  David Goldberg  (Palo Alto Research Center)
Automated analysis of glycan spectra

9:00 – 9:20  Minoru Kanehisa  (Kyoto University)
Integration of genomic, chemical, and pathway information for glycoinformatics

9:20 – 9:40  Hisashi Narimatsu  (National Institute of Advanced Industrial Science and Technology)
Enzymatic synthesis and high throughput structural analysis of glycans using glycogene library

9:40 – 10:05  MORNING BREAK

10:05 – 10:25  Claus-W. von der Lieth  (German Cancer Research Center)
Informatics in glycomics: status and perspectives

10:25 – 10:45  James C. Paulson  (The Scripps Research Institute)
Activation-induced changes in N-linked glycosylation of T lymphocytes

10:45 – 11:00  Oliver Garden  (Imperial College London)
Glycotranscriptomic and glycomic analysis of CD25+ and CD25- CD4+ T cells in murine models of systemic lupus erythematosus: preliminary findings

11:00 – 11:15  Thomas Scanlin  (Robert Wood Johnson University Medical Group)
CFTR, glycosylation and glycosyltransferase distribution

11:15 – 11:30  Pauline Rudd  (University of Oxford)
Detailed analysis of the O-glycosylated regions of secretory IgA1 and gelatinase B using HPLC/MS based strategies combined with the GBI oligosaccharide structural database provides insights into immunity and metastasis

11:30 – 11:45  Risto Renkonen  (University of Helsinki)
A semi-automated approach to detect N-linked glycopeptides

11:45 – 1:45  LUNCH BREAK (lunch to be provided)
POSTERS at the Atrium area
DATABASE DEMONSTRATION 2As.10 conference room

12:15 to 1:15 pm  SUBGROUP MEETINGS at the Natcher Building (bring your lunch if desire)
Siglec Subgroup – 2AN24k conference room
TCR Subgroup – 1As13 conference room
Galectin Subgroup – 3An.34 conference room
C-type lectin Subgroup – 3As.13 conference room
Other Subgroup – in room Balcony B
1:45 – 3:05   GLYCANS AS CELL SURFACE RECEPTORS

1:45 – 2:05   Irwin Goldstein (University of Michigan)
             The glycomics array: A tool for determining the carbohydrate binding specificity of lectins

2:05 – 2:20   Mavis McKenna (University of Florida)
             Sweet talk: Biophysical characterization of virus capsid–host interactions

2:20 – 2:35   Willie Vann (Center for Biologics Evaluation and Research / FDA / NIH)
             Interaction of Clostridium neurotoxins with gangliosides

2:35 – 2:50   Peter Lipke (Hunter College CUNY)
             Roles for N-, O- and GPI-glycosylation in secretion, cell wall cross-linking, and activity in fungal adhesins

2:50 – 3:05   Senitiroh Hakomori (University of Washington)
             Functional glycomics in search for carbohydrate epitope which binds specifically to carbohydrate, as basis of cell-cell interaction

3:05 – 3:30   AFTERNOON BREAK

3:30 – 4:50   C-TYPE LECTIN

3:30 – 3:50   Kurt Drickamer (Imperial College London)
             Glycan arrays as a bridge between glycomics and receptor biology

3:50 – 4:10   Minoru Fukuda (The Burnham Institute)
             Novel findings on structure and function of L-selectin ligands

4:10 – 4:30   Yvette van Kooyk (VU Medical Center)
             C-type lectins on DC, glycan specificity and immune function

4:30 – 4:50   John Lowe (University of Michigan Medical School)
             Glycan-dependent control of cell adhesion and signal transduction

4:50 – 5:30   WRAP-UP/SUMMARY/FAREWELL

     Subgroup Leaders: P. Crocker, R. Cummings, K. Drickamer, M. Kronenberg, J. Paulson
     NIGMS: P. Marino

6:30 p.m.    Database Roundtable meeting at the Marriott Hotel in the Annapolis/Chesapeake room
             (invited guests only)
**Consortium for Functional Glycomics**  
**Participating Investigator Meeting, May 17-18, 2005**

**POSTER SESSION** from 11:30 to 1:30 at the Atrium area

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INCREASED SIALYLATION AND REDUCED LACTOSAMINE EXPRESSION IN BREAST CANCER CELLS REDUCES CROSS-LINKING OF GELATINASE B TO ECM PROTEINS BY GALECTIN-3

Simon Fry¹, Louise Royle¹, Philippe van Den Steen², Mark Wormald¹, David Harvey¹, Anthony Leathem³, Raymond Dwek¹, Ghislain Opdenakker² and Pauline Rudd¹

¹Oxford Glycobiology Institute, Oxford, UK; ²REGA Institute for Medical Research, Leuven, Belgium; ³Royal Free and University College London Medical School, London

Gelatinase B (MMP-9) is a heavily glycosylated member of the matrix metalloprotease family of Zn-dependent endopeptidases. Expression is increased in many cancer cell types, including breast cancer. Experimental metastasis assays demonstrate that gelatinase B is required for effective cell invasion and metastasis. Gelatinase B substrates are extracellular matrix proteins including gelatins, collagens, aggregan and importantly galectin-3. The galectin-3 carbohydrate recognition domain (CRD) binds galactosides and shows a dramatic increase in affinity in response to increases in repeating lactosamine units in its ligands. Galectin-3 cross-links glycans (and therefore ECM proteins such as laminin) by self-associating to form dimers-pentamers through its N-terminal domain, a property lost following cleavage by gelatinase B.

The N- and O-linked glycans of native human neutrophil gelatinase B have been sequenced. 51% of the clustered O-glycans (14 potential O-glycosylation sites in the 56 amino acid collagen V-like domain) are bound by galectin-3, with 11% containing a di-lactosamine repeat and 4% a tri-lactosamine repeat. We analysed the glycosylation of gelatinase B secreted from MCF-7 breast cancer cells. Only 8% of the O-glycans are bound by galectin-3, and there are no lactosamine repeats. The major difference in N-glycosylation was the presence of a 1-3 and a 1-2 linked outer arm fucosylation in neutrophil and MCF-7 gelatinase B respectively.

Our binding studies demonstrate that aberrant glycosylation of breast cancer associated gelatinase B results in significant reduction in galectin-3 CRD binding. Altered gelatinase B glycosylation in breast cancer cells can thus enhance metastasis as diffusion through the ECM is increased as a result of a reduction in binding to galectin-3, and therefore to laminin.

APPLICATION OF STATE OF THE ART GLYCAN SEQUENCING TECHNOLOGY TO O-GLYCANS FROM BREAST TUMOUR TISSUE

Fry, S.A.¹, Royle, L.¹, Dwek, M.V.², Leathem, A.J.C.², Dwek, R.A.¹, Rudd, P.M.¹

¹Glycobiology Institute, Department of Biochemistry, University of Oxford, ²Department of Surgery, Royal Free and University College London Medical School, Institute of Surgical Studies.

There have been many reports of aberrations in breast cancer glycosylation. These may influence cancer cell behaviour by enabling cellular interactions that promote metastasis or by facilitating tumour cell evasion from immuno-surveillance. Characterisation of glycans from breast tumour tissue is required to understand the role that altered glycosylation plays in the pathogenesis of this disease. The O-linked glycans elevated in breast cancer being characterised following their release from ~2mg of tissue by optimised hydrazinolysis. The glycans are fluorescently labelled and analysed by a combination of normal phase and weak anion exchange HPLC before and after a range of specific exoglycosidase digestions. Structures are assigned from these data by reference to an O-link database constructed by our group.
Complete N- and O-glycan analysis of Human Secretory IgA, reveals substantial differences in glycosylation between the J chain, secretory component, and IgA heavy chains.

1L Royle, 1DJ Harvey, 1MR Wormald, 1RA Dwek, 1PM Rudd.

1Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, United Kingdom.

Secretory IgA (SIgA) consists of two monomeric IgA units and two additional polypeptide chains, the J chain and Secretory Component (SC). The H, L and J chains are synthesized and assembled into dimeric IgA in plasma cells, whereas SC is added during transport across the mucosal epithelia.

The N-and O-glycans of the individual peptides from human colostrum SIgA were identified using a sensitive sequencing strategy in which the released glycans were fluorescently labeled and analysed by a combination of HPLC, exoglycosidase digestions, MALDI-MS and LC-MS/MS.

SIgA1 has several sites capable of binding to pathogens, which can block their attachment and subsequent infection of mucosal surfaces. In addition to 4 Fab sites that can bind to specific antigen epitopes, there are 2 O-glycan regions (with up to 20 glycans per region), and a SC region (with 7 N-glycans), all of which present complex fucosylated and sialylated glycan epitopes capable of interaction with bacterial adhesins. In contrast, the N-glycans on the H chain are mainly short non-galactosylated structures, whilst the J chain has a single sialylated N-glycan.
Abstract

The steps involved in the transfer of mannoproteins with glycosyl-phosphoinositol (GPI) phospholipid anchors from the plasma membrane to the cell wall are unknown. The process involves the loss of phospholipid, inositol, and glucosamine followed by transglycosylation of the GPI-anchor remnant oligosaccharide to b1,6 glucan. b1,6 glucan is, in turn, anchored to b1,3 glucan of the inner cell wall. All of these reactions are external to the cytoplasm and must be catalyzed by proteins identified as membrane, "periplasmic," or cell wall proteins.

Isolation of intact delipidated GPI-anchor remnants is the first step in preparing affinity probes or columns to identify, isolate, and characterize GPI-binding and -processing proteins. For this task, Cwp2p was selected to be the carrier protein. Because Cwp2p is the smallest protein that is targeted to the cell wall, it would have the highest molar yield of GPI-anchors per milligram of protein. The HA-tagged Cwp2p protein was successfully modified to have a unique trypsin cut site and a unique cysteine residue near the C-terminal to facilitate identification and recovery of the GPI-anchor peptide after proteolytic cleavage of the HA-tagged Cwp2 protein. Immunological assays confirm synthesis, anchorage, and excretion of HA-tagged Cwp2p when transformed cells are grown in galactose media. Labeling with biotin-maleimide confirms the presence of the cysteine residue in the modified Cwp2p sequence.
Bioinformatics of High Serine-Threonine Sequences

Juan Coronado, Susan Epstein, Oliver Attie, Wei-Gang Qiu, and Peter Lipke
Depts. of Biology and Computer Science, Hunter College

**Background:** Although low-complexity sequences comprise a significant portion of the proteome, reliable and accurate homology detection among them remains elusive. Sequence comparison methods, such as BLAST and FASTA, use scoring matrices optimized for elucidating homology among sequences with diverse amino acid compositions, i.e., high Shannon entropy. When identical residues align in non-homologous positions, low-complexity sequences align with anomalously high scores. This phenomenon, known as low-complexity corruption, generates false alignments with inappropriately low e-values. A reliable approach to the alignment of such sequences is essential for structural and evolutionary studies of the low-complexity part of the proteome.

**Results:** Pairwise comparisons of a low-complexity, yeast cell-wall protein set with composition-based modification of scoring matrices show an improvement in sensitivity and selectivity over standard BLAST or FASTA comparisons that use BLOSUM62. Using six (6) different scoring matrices, searches of the *Saccharomyces cerevisiae* genome identify a consistent set of similar sequences, and reject randomized sequences and non-homologous ORFs in sensitivity tests and in transitive closure tests.

**Conclusion:** Selective reduction of scores for high-frequency residue pairs yielded searches with good sensitivity and maximal discrimination for these low-complexity sequences.

Supported by RCMI
Glycosylated Tandem Repeats in the *Candida albicans* Adhesin Als5p Affect Structure and Function

Henry Otoo, Jason Rauceo, and Peter N. Lipke
Dept. of Biology and The Center for Gene Structure and Function, Hunter College CUNY, New York, NY.

Fungal infections have increased as a result of new surgical procedures, AIDS and diabetes. *Candida albicans* is responsible for most of these infections, including oral, vaginal, and disseminated infections. *C. albicans* Als adhesins (which are covalently bound to the surface of cell walls) are involved in binding to the host, a first step before colonization and pathogenesis. The N-terminal Ig-like regions have been shown to mediate binding to endothelial cells and Fibronectin–coated beads. Als proteins also have a central tandem 36-residue repeat motif (TR region) that is rich in threonine and other beta-branched amino acids. The TR repeats vary in number from one Als protein to another.

We report that the TR-region of Als5p is O-glycosylated. Circular dichroism indicates that the Ig region is rich in beta sheets (Sheppard *et al.* 2004, Hoyer *et al.*, 2001). In contrast, the TR-region is rich in _-helical and unordered structures. An ELISA assay shows that the TR region improves binding of the Ig-region to fibronectin.

TR-like tandem repeats are common to the Als proteins and also in other fungal adhesins and flocculins, so they are good potential targets for development of anti-fungal drugs.

Supported by NIGMS-SCORE
Automated interpretation of MS/MS spectra of oligosaccharides

Haixu Tang; Yehia Mechref; Milos V. Novotny; Indiana University, Bloomington, IN

Keywords: Computational Methods; Computer Program; Oligosaccharides; Post Source Decay;

Novel Aspect: A novel dynamic programming algorithm for automated interpretation of MS/MS spectra of oligosaccharides, taking into consideration the cross ring fragmentations.

Introduction
The emerging glycomics and glycoproteomics projects aim to characterize all forms of glycoproteins in different tissues and organisms. Tandem mass spectrometry is the key experimental methodology for high throughput glycan identification and characterization. Fragmentation of glycans from high energy collision-induced dissociation (CID) generate ions from glycosidic as well as internal cleavages. The cross-ring ions resulting from internal cleavages provide additional information that is important to reveal the type of linkage between monosaccharides. This information, however, is not incorporated by the current programs for analyzing glycan mass spectra. As a result, they can rarely distinguish isomeric oligosaccharides from the mass spectra, which have the same saccharide composition, but different types of sequences, branches or linkages.

Methods
We describe a novel algorithm for glycan characterization using tandem mass spectrometry (MS/MS). Our strategy is based on finding and scoring potential prefix residue features (PRFs), by evaluating the local fragmentation patterns; and then trying to deduce the oligosaccharide structure from these PRFs by dynamic programming algorithm. The entire algorithm consists of three steps. First we develop a scoring scheme to identify potential bond linkages between monosaccharides, based on the appearance pattern of cross-ring ions. Next, we adopt a dynamic programming algorithm to determine the most probable oligosaccharide structures from the mass spectrum. Finally, we re-evaluate these possible oligosaccharide structures, taking into account the double fragmentation ions.

Preliminary results
The algorithm described above is implemented in a program entitled GLYCH (GLYcan CHaracterization), written in C language on UNIX/LINUX system. We have tested this program for several experimental MS/MS spectra of oligosaccharides, obtained by MALDI/TOF/TOF mass spectrometry. All MS/MS spectra were preprocessed to remove redundant and noisy peaks before being analyzed by GLYCH program. The results showed that in all cases, the real structure is among the best solutions GLYCH identified. The dynamic programming (DP) approach can identify the real oligosaccharide among many other ones which have very similar structure, often differing by only one linkage. The re-evluation procedure can dramatically reduce the possible optimal solutions, while keeping the real structure among the optimal solutions. For more complicated structures, e.g. the oligomannose with branching structure, the real oligosaccharide is not ranked best by DP approach; after the re-evaluation, however, it is among the optimal solutions.
A Sweet Treat to Target the Breast Cancer Cell for Therapy

Abstract:
Carbohydrate receptors are present in all cells in order to initiate and participate in a variety of cell functions. Tumor cells have been found to have increased expression of some of the beta- Galactose binding molecules (galectins). These molecules are known to play an important role in cell growth, adhesion, migration and transformation, tumor metastasis, formation of cysts, as well as being elevated in some human tumors. Galectin-3 is one of these galectins that has been found to be upregulated in breast tumor epithelium, inhibiting and protecting these cells from intrinsic apoptosis due to their complex involvement in cell adhesion.

Our hypothesis is that due to the altered expression of the galectin family of molecules in tumor cells, there will be a proportionally increased uptake of a beta-galactoside linked compound in tumor cells and this uptake can be used to target drugs to the tumor cells. The carbohydrate-lectin interactions between a multivalent beta-lactose compound and tumor or normal cells were evaluated via glycohistochemical analysis of the biotinylated saccharide with histological sections of tumor and normal tissues. The absence or minimal occurrence of carbohydrate uptake by normal cells will enable carbohydrates to be attached to chemotherapeutic agents and be administered to cancer patients, resulting in therapeutic effects at lower doses of chemotherapy with lower side effects.

Evaluations have begun on tissue microarray slides from NIH. These microarrays contain hundreds of sections of normal and tumor tissues. The microarray slides have been prepared differently and require assay modifications of increased rehydration and dehydration in order to be effectively analyzed. Initial staining of breast cancer sections on slides indicates that multivalent lactose will bind to these tissues at an increased rate.
Metabolic pathways of natural glycolipid ligands for NKT cells in tumor immunity

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ABSTRACT

NKT cells function as a paradoxical cell type in tumor immune surveillance through acting on antigen presenting cells, natural killer cells, and CD8 T cells. Alpha-galactosylceramide (αGalCer), a marine sponge glycolipid, induces Th1 cytokine production by NKT cells, and prevents metastasis in some mouse experimental systems. In a methylcholanthrene (MCA) induced mouse primary fibrosarcoma system, endogenous lipids activate NKT cells to prevent tumor progression. However, in transplanted tumor systems, NKT cell activated by endogenous lipids promote tumor growth by producing Th2 cytokines specifically IL13 (see cartoon for Th1 versus Th2 activation).

To identify the mechanism explaining the above paradoxical functions of NKT cells, we have tried to characterize the natural lipid ligands of NKT cells. We have used genetic approaches including gene targeted mice and RNAi silenced cell lines to dissect the pathways (see schemes of synthesis and degradation) involved in the trafficking, processing and loading of glycolipids onto CD1, an MHC-like glycoprotein family specialized in microbial and self lipid antigen presentation. We found that the development and the function of CD1/lipid specific T cells are critically regulated by enzymes (β-hexosaminidases) and activator proteins (saposins) of glycolipid metabolism. This led us to narrow down the natural glycolipid antigens to substrates of these enzymes. By studying those glycolipids candidates, both chemically synthesized, and purified from natural sources, we first identified the isoglobotrihexosylceramide (iGb3) as a lysosomal endogenous ligand for T cells (see figure1). Genetic evidence suggests that natural ligands must be products of β-hexosaminidases (figure 2). Thus iGb3 might be the only natural ligand for NKT cells based on our current knowledge on structures of mammalian glycolipids.

Ongoing studies are focused on the identity of the natural NKT cell ligands in tumor models and the metabolic pathways in generating these glycolipid ligands. We intend to identify the natural lipids responsible for the beneficial or detrimental activation of NKT cells, and the subtle changes in either lipid part or carbohydrate part that might cause switching of cytokine production profiles. With regard to the detrimental activation, we intend to find approaches to reverse the Th2 (IL13) cytokine production profile, by introducing exogenous glycolipid ligands that induce Th1 cytokine production. In collaboration with synthetic chemists, we will use the αGalCer and iGb3 glycolipids as the templates to design better structures that might possess therapeutic function.
Interaction of Mannose Binding Lectin with Viruses

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ABSTRACT
Since complement plays a role during infection by certain viruses, we assessed the ability of Mannose Binding Lectin (MBL) to interact with and neutralize viruses containing envelope glycoproteins from Ebola virus, Marburg virus, human immunodeficiency virus (HIV), vesicular stomatitis (VSV). Cytomegalovirus was also evaluated in some experiments. HEK 293 cells were co-transfected with expression plamids for either Ebola, Marburg, VSV or HIV envelope proteins along with a plasmid encoding structural proteins for HIV resulting in production of virions pseudotyped with the different envelope proteins. All viruses except VSV bound efficiently to MBL. MBL blocked the ability of ebola-pseudotyped virus to bind to DC-SIGN. MBL did not efficiently neutralize filoviruses, HIV or CMV alone. However, addition of MBL to serum neutralized ebola-pseudotyped virus. Different clades of HIV appeared to have somewhat different binding to MBL with clade B viruses binding more frequently.
Since MBL can activate complement and direct opsonization, our studies suggest that numerous types of viruses, either during disease or during virus-mediated gene therapy, could be cleared by MBL.
P-SELECTIN CONTROLS IMPORTATION OF THYMIC PROGENITORS

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Thymic homeostasis in adult mice is maintained by gated importation of circulating T cell precursors (CTPs). The mechanisms controlling thymic homing of CTPs are poorly understood. We have recently found that P-selectin and its ligand PSGL-1 are important in the control of thymic precursor homing and importation. Indeed low level of constitutively expressed P-selectin can be detected by histochemistry and P-selectin binding is detected on bone marrow CTPs and on DN1/DN2 but not on DN3/DN4 thymocyte subsets. To gain a better insight into the underlying mechanisms we used real time PCR to study thymic P-Selectin mRNA levels.

Real Time PCR procedures were established to measure the relative transcript levels for P-selectin in wild type and IL7Rnull mice. To normalize the P-selectin mRNA levels for the thymic endothelial cells we used Cadherin 5, exclusively expressed by endothelial cells, as a reference gene. We found 9 fold higher levels of P-selectin mRNA in IL7Rnull mice as compared to wild type mice. After i.v. injection of prothmocytes we measured a 6 fold reduction of thymic P-selectin transcripts in reconstituted IL7Rnull mice compared to non-reconstituted IL7Rnull. Furthermore these levels of thymic P-Selectin mRNA were comparable to levels of P-selectin mRNA expression in the wild type controls.

The downregulation of P-selectin mRNA expression might indicate a negative feedback mechanism for P-selectin expression controlled by the occupation status of the intrathymic niches.

Research is funded by a grant from CIHR.
Carbohydrate-binding characteristics of human C-reactive protein

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Abstract
Human C-reactive protein (CRP) is a proto-type acute phase reactant, which is often used to gauge the presence of inflammation. CRP can eliminate some microorganisms in a fashion similar to IgG, by initiating classical complement cascade after binding to multivalent ligands. Structurally, CRP belongs to pentraxin family, having five monomers associating non-covalently in a ring form. One face of pentameric ring contains all the calcium-binding sites, two sites per monomer in close proximity to each other. Phosphoryl choline (PC), the best ligand for CRP, is bound near the calcium site, with phosphate group directly coordinating with both calcium atoms. Conformation in the calcium-binding area undergoes change depending on the presence or absence of calcium. In addition, there is a slightly denatured, but physiologically important, form of CRP, known as neoCRP or mCRP, which is formed, for instance, when CRP is adsorbed onto a plastic surface.

CRP binds at least three types of ligands: 1) PC and related structures; 2) poly-cationic compounds, such as poly-lysine and protamine sulfate; 3) carbohydrates containing galactose and related structures. We developed ligand-binding assays based on europium-labeled reporter ligands that are applicable to all three ligand groups. The native CRP assay uses polyethyleneglycol-assisted precipitation of CRP—ligand complex, and neoCRP assay is carried out with CRP that is immobilized onto 96-well microplate. The following major conclusions were obtained from direct and inhibition assays using europium-labeled PC-BSA, poly-L-lysine, lactose-BSA, Gal6P-BSA, and Galb(1,3)GalNAc-BSA.
1) Binding of PC requires calcium, while neutral sugars are bound in the absence of calcium. Polycations are bound with or without calcium.
2) Gal6P is the best carbohydrate ligand, and it can be bound with or without calcium. PC and Gal6P share the same phosphate-binding site, with choline and galactose moieties occupying either side of phosphate. Binding of Gal6P in the presence of calcium is approximately 10-fold stronger than in the absence of calcium.
3) Neutral sugars are much weaker ligands than Gal6P. However, we showed that BSA conjugate containing Galb(1,3)GalNAc (one of the best disaccharide inhibitors) has sufficient affinity to be bound to both native CRP and neoCRP. Gal6P is the best inhibitor for the binding of Galb(1,3)GalNAc-BSA.
4) Unlike PC which is bound in a wide pH range, binding of carbohydrate ligand, whether neutral or phosphorylated, has pH optimum around 6.5 above which binding decreases steadily, suggesting that the binding site for galactose and disaccharides becomes masked above neutral pH.
5) Protein structure of neoCRP appears to be more fragile than native CRP in the acidic region. Binding of all ligands, including PC-BSA, declined in a parallel fashion below pH 6, having no binding activity left at pH 4.5. In contrast, native CRP maintains active conformation down to pH 4.5.
Detection of neoplasia-specific clusters of anti-glycan antibodies in sera of breast cancer patients using a novel glycan array

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**Background:** Malignant transformation and tumor progression are associated with the specific and significant changes in the complex surface carbohydrates, known as Tumor-Associated Carbohydrate Antigens (TACAs). Production and presence of antibodies against these abnormal carbohydrates in the human blood during cancer progression is expected. A robust glycan array has been recently fabricated (Blixt et al, 2004, *PNAS*, 101, 17033) that employs a library of over 200 well-defined glycan structures. We tested the hypothesis that this glycan array can be used to detect anti-glycan antibodies in human serum from patients with metastatic breast cancer (MBC).

**Results:** A total of 18 patients with MBC evaluated at the MDACC between 7/01 and 8/02 were analyzed. The predominant site of disease was visceral (61%). The median survival was 15+ months (range 3-39+). The median follow-up was 33 months. As control, or “healthy” sera, we have used sera from 15 healthy individuals with no known malignancies. Patients with MBC demonstrated 2-10-fold higher levels of several antibodies against specific glycans including: N-acetyllactosamines, fucosylated, sulfated and Core-2 glycans. Antibodies against ceruloplasmin and the cancer-specific carbohydrate antigen Neu5Ac\(^{2-6}\)GalNAc\(^{2}\) (STn), show most significant higher presence in all patients with MBC as compared to healthy individuals. A mathematical simulation model for the data analysis is being developed.

**Conclusion:** Our studies show that the printed glycan array is a sensitive and promising tool in the development of clinical serum-based screening test for neoplasia based on the simultaneous detection of the tumor-specific clusters of circulating anti-glycan antibodies. We are in a process of validating these results in the larger cohort of patients with MBC, and evaluating the presence of circulating anti-glycan antibodies in patients with BC stage I-III, and patients with the benign breast disease with high risk of progression to malignancy.
**Synthetic lactulose amines bind tumor-promoting galectins –1 and –4, and inhibit breast cancers in Her-2/neu transgenic mice.** Huflejt M, Mossine V, Naidenko O, Jazayeri M, Rogers P, Tinari N, Iacobelli S, Elliott M, Lustgarten, J, Croft M. Sidney Kimmel Cancer Center, San Diego; U. of Missouri, Columbia; LIAI, San Diego; Universita G. D'Annunzio, Chieti, Italy; Scripps Clinic, La Jolla, California.

The transition of cells from normal to malignant is associated with significant changes in the expression of complex surface carbohydrates and carbohydrate-binding proteins. We have observed significant increases in the expression of $\beta$-galactoside binding proteins, galectins –1 and –4, during the progression of human breast disease. We theorize that these carbohydrate-binding proteins are not just by-standers, but play an integral part in the cancer process: 1) immunosuppressive properties of galectin-1 have been reported in autoimmune diseases, 2) tumor-promoting activities of galectin-3 are well known, and 3) we have noted anti-apoptotic activity of intracellular galectin-4. These observations prompted us to search for galectin inhibitors, which could be critical in disrupting the cancer process.

**Methods and Results:** Using a surface plasmon resonance galectin binding assay, we screened a panel of synthetic, small, non-toxic and non-hormonal lactulose amines, and identified one compound, L2hmda, which binds galectins-1 and –4 with affinities relatively high for protein-carbohydrate interactions. L2hmda not only inhibits binding of galectin-1 to its extracellular physiological ligands 90K/MAC-2BP and laminin in ELISA assays, but more importantly, L2hmda blocks the immunosuppressive activity of galectin-1 on T-cells *in vitro*. To demonstrate the *in vivo* relevance of these observations, we tested L2hmda for its anti-tumor activity in Her-2/neu transgenic mice. These mice spontaneously develop human-like lobular carcinomas, which are notoriously difficult to inhibit. Despite the fact that these mice are heavily immunosuppressed, this galectin inhibitor markedly reduced the growth of tumors when used alone, and nearly completely eradicated tumors when used in conjunction with dendritic cell-based immunotherapy.

**Conclusion:** Our studies are the first to show that simple glycoconjugates can inhibit galectin immunosuppression *in vitro* and tumor growth *in vivo*, with a potential future role in cancer prevention.
Protection against diarrhea by breastfeeding is often attributed to the secretory antibodies of human milk. However, we found that 1,2-linked fucosylated oligosaccharides (FucOS) and other glycans (FucG) of human milk strongly inhibit host ligand binding to several diarrheagenic agents. We investigated the relationship between maternal Lewis blood group type and FucOS expression in milk, and between variable FucOS expression and risk of diarrhea in their infants, in a cohort of 93 Mexican breastfeeding mother-infant pairs. HPLC of the milk revealed eight major oligosaccharide peaks, each corresponding to one of the major Lewis antigens. Expression of 1,2-linked FucOS in human milk varies by maternal Lewis blood group type, consistent with their synthesis by fucosyltransferases of the secretor (FUT2) and Lewis (FUT3) gene family. Thus, the FucOSs of milk are produced constitutively and vary among mothers. As in our previous laboratory studies, specific fucosylated moieties of glycans inhibit specific pathogens: Relatively high quantity of 2'-fucosyllactose (H-2) in milk was associated with protection against infant diarrhea due to campylobacter (n=31 cases, p=0.004) and relatively high quantity of lacto-N-difucohexaose I (Le(b)) was associated with protection against diarrhea due to norovirus (n=16 cases, p=0.012). Infants who developed ST-associated diarrhea were consuming milk with lower relative amounts of 2-linked FucOS than the remaining infants (p<0.001). Infants consuming milk high in 1,2-linked FucOS had significantly lower relative risk of moderate-to-severe diarrhea of all causes (p=0.001). Thus, human milk FucOS are produced constitutively, contain the Lewis epitopes, and are strongly associated with lower risk of enteric disease. We propose that specific FucGs, including FucOSs, are major constituents of an innate immune system of human milk whereby the mother protects her infant from enteric and other pathogens.
**Increased Susceptibility of Secretor Blood Group Fucosyltransferase FUT2-Null Mice to Experimental Vaginal Candidiasis**

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**Objective:** We developed a mouse model of the human non-secretor to study the role of a common gene polymorphism in recurrent vulvovaginal candidiasis. Approximately 20% of humans have a frame-shift mutation in the *Secretor gene (FUT2)*, encoding an ABH histo-blood group a(1,2)fucosyltransferase (E.C. 2.4.1.69) that elaborates a(1,2)fucose residues on mucins and epithelial of the gastrointestinal and reproductive tracts. *Candida albicans* is the major causative agent of vulvovaginal candidiasis affecting up to 75% of women in their lifetime; moreover, up to 10% of these women suffer from recurrent episodes. Epidemiological evidence indicates an increased susceptibility to recurrent infection in women with a *FUT2* non-secretor mutation.

**Methods:** To explore the predicted role of *FUT2* mutation in susceptibility to vaginal candidiasis, Fut2-LacZ knockout reporter mice were studied using whole organ X-gal staining, lectin histochemistry, and an estrogen-dependant experimental model of yeast vaginitis.

**Results:** By whole organ X-gal staining, Fut2-LacZ activity is highly expressed in glandular endocervix, but not vaginal epithelium. Similar to the hormonal regulation of Fut2 expression in uterus, endocervical Fut2-LacZ expression varies with the estrous cycle and is highest in estrus. Upon lectin staining, a(1,2)fucosylated glycans are present in endocervix and superficially in the vaginal lumen of wild type mice, but absent in null mice. Susceptibility of Fut2-LacZ null mice to *C. albicans* infection was examined in an estrogen-dependant model of yeast vaginitis. Fut2-LacZ null mice display an increased susceptibility to vaginal yeast colonization compared to wild type mice (1-way ANOVA, P<0.001). Pre-incubation of yeast prior to vaginal inoculation with simple sugars conjugated to albumin affects colonization rates differentially between wild type and null mice suggestive of a carbohydrate-microbe adhesion mechanism.

**Conclusion:** These data support the importance of *FUT2* in mucosal immunity and demonstrate a similar phenotype between Fut2-LacZ null mice and human non-secretors in host-microbe interaction.
Characterization of the Mechanism of Acquired Virulence by Minute Virus of Mice Parvovirus Using a Glycan Array and BIACORE Surface Plasmon Resonance

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The prototypic strain of the autonomous murine parvovirus, minute virus of mice (MVMp), differs from the immunosuppressive strain (MVMi) in both host cell range and pathogenicity. While MVMi is lymphotropic and can cause a lethal infection for certain genetically inbred mice and adult mice with severe combined immunodeficiency (SCID), MVMp possesses a fibrotropic host cell preference and causes an asymptomatic infection in permissive hosts. Several mutants of MVMp were recently isolated in SCID mice, each with a single amino acid change, at either position 325, 362 or 368, in their major capsid protein VP2, capable of causing a lethal infection in SCID mice. The MVMp mutations are found close to (325) or on the capsid surface (362 and 368), with the later two residues being adjacent to a sialic acid binding site recently mapped onto the MVMp capsid at the icosahedral 2-fold axes (2F). Pretreatment of host cells with neuraminidase has shown that sialic acid is an important component of the cell surface recognition and infection by MVM, thus we investigated the role of receptor interaction(s) on the pathogenic phenotype of MVM capsids. We used a glycan array, developed by cores D and H of the Consortium for Functional Glycomics, containing ~180 different carbohydrates, to identify the sialic acid structures recognized by the wt MVM and mutant capsids. Surface plasmon resonance bio-specific interaction assays were used to independently confirm the binding of the capsids to the sugars identified in the array and to determine the relative affinity of the MVM capsids. To determine the contribution of relative binding affinity to virulence determination for the wt MVMp and mutant capsids in the context of a sialoglycoprotein glycophorin A was used in Biacore assays because the MVMp infectious receptor is unknown.
ELICITATION OF ANTI-MANNOSE ANTIBODIES AND THEIR CROSS-REACTIVITY TO HIV-1 ENV GLYCOPROTEINS

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Human immunodeficiency virus 1 (HIV-1) Env gp120 glycoprotein contains more than 20 glycosylation sites. Approximately half of them are high mannose and hybrid types of carbohydrates. One of the few broadly neutralizing monoclonal antibodies isolated from HIV-1 infected humans, 2G12, recognizes a carbohydrate epitope composing of high mannoses on gp120 glycoproteins. HIV-1 binding to dendritic cells was found through the interaction of high mannoses on HIV-1 Env glycoprotein with the cell surface molecule of DC-SIGN on dendritic cells. Thus, antibodies specific to high mannose carbohydrates are of interest in preventing HIV-1 infection. In this study, antibodies against mannose were elicited in rabbits with a yeast cell wall extract zymosan A that contains glucan and mannan, while yeast mannan alone failed to do so. This could be due to the activation of different Toll like receptors (TLR) by zymosan and mannan. Zymosan could not induce antibody response in mice. Analysis of a large number of the third complementarity-determining regions of immunoglobulin heavy chain variable region (CDR-H3) showed that there is a significant difference not only in the length but also in the diversity of amino acid sequence between the two species in their CDR-H3 regions. The immune serum from rabbit reacted with gp120 glycoproteins from different HIV-1 strains and isolates in a wide range of sensitivity. Mannan- and mannos-specific antibodies and total IgG were further isolated from immune sera by affinity chromatography. The activity of anti-mannan and anti-mannose IgG to mannan was significantly higher than that of total IgG and whole serum, while they did not show cross-reactivity to gp120 glycoproteins. The total IgG isolated by protein G showed a stronger cross-response to the gp120 glycoproteins in subtypes C and E than serum and the mannan and D-mannose purified antibodies. These results indicate that there is a very small fraction of antibodies in the immune serum that reacts with the carbohydrates on HIV-1 Env glycoproteins and that needs to be further isolated from the pool of antibodies with different specificities.
Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by the systemic deposition of immune complexes. Central tolerance appears to be intact in lupus, suggesting that aberrant peripheral tolerance plays a role in its pathogenesis. Mice of the MRL/Mp strain were studied as a polygenic model of SLE that closely reflects the human disease. While MRL/Mp CD4⁺CD25⁺ regulatory T cells (Tregs) displayed only subtle abnormalities of regulatory function \textit{in vitro}, CD4⁺CD25⁻ T cells showed significantly reduced sensitivity to suppression by Tregs \textit{in vitro}, as determined by cross-over experiments in which MRL/Mp CD4⁺CD25⁻ T cells were cultured with CBA/Ca CD4⁺CD25⁺ Tregs.

Recent studies involving Mgat5⁻/⁻ and _α-mannosidase II⁻/⁻ mice implicate a role for glycomic lesions in the pathogenesis of lupus. We hypothesized that glycomic lesions contribute to the abnormal functional interactions between MRL/Mp CD25⁻ and CD25⁺ CD4⁺ T cells promoting defective peripheral tolerance and disease.

Using Core E consortium resources we investigated the glycotranscriptomes of T cells from lupus-prone and control mice. Preliminary studies have yielded the first of a triplicate set of data. Interesting leads include proteins involved in the cell cycle, apoptosis, cell migration and T cell development. Definitive conclusions await triplicate analyses.

The MALDI-TOF spectra of N-glycans from CD4⁺CD25⁺/⁻ T cells from BALB/c mice selected by Operator 1 revealed a high prominence of biantenary, NeuGc2-core fucosylated structures. In comparison, identical experiments conducted by Operator 2 revealed a high prominence of biantennary, NeuAc2, with no core fucose and triantennary NeuAc2, with no core fucose. Thus, suggesting that the CD4⁺ T cell glycome is exquisitely sensitive to subtle differences in the way cells are prepared by different operators.

On-going studies are attempting to dissect the molecular mechanisms of defective peripheral tolerance in the MRL/Mp mouse.
Poster #6001

Subunit sialic acids differentially alter cardiac and neuronal voltage-gated sodium channel subunit gating

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Previously, we showed that sialic acids (SA) attached to the sodium channel (Na\textsubscript{v}) \(\beta\) subunit are important for modulation of Na\textsubscript{v} gating (Johnson et al., J. Biol. Chem. 2004). We wished to determine if SA attached to the Na\textsubscript{v} \(\alpha\) subunit could also modulate channel gating. Thus, \(\beta\) was co-expressed with the neuronal \(\alpha\) subunit, Na\textsubscript{v}1.2, or the cardiac isoform, Na\textsubscript{v}1.5, in the fully sialylating Pro5 and essentially non-sialylating Lec2 cell lines. For Na\textsubscript{v}1.5, \(\beta\) caused a SA-dependent hyperpolarizing shift in the voltage-dependence of activation (\(V_a\)) but had no significant effect on the voltage dependence of inactivation (\(V_i\)). Interestingly, \(\beta\) caused an SA-independent depolarizing shift in \(V_a\) and \(V_i\) for Na\textsubscript{v}1.2. Next, the effects of \(\alpha\) and \(\beta\) sialic acids on Na\textsubscript{v}1.2 or Na\textsubscript{v}1.5 gating were studied, and appeared to be additive. That is, the gating effects of \(\alpha\) and \(\beta\) on Na1.5 were additive, but only when SA was attached to the \(\beta\) subunits. In the absence of SA, the \(\beta\) subunits were unable to modulate gating of Na1.5. There was essentially no observed effect on Na\textsubscript{v}1.2 gating by \(\alpha\) and \(\beta\) in the presence of SA. However, when sialylation was prevented, the \(V_a\) and \(V_i\) were shifted in the depolarized direction similarly to that observed when only \(\beta\) was expressed with Na\textsubscript{v}1.2. Here we show that \(\beta\) can impose SA-dependent and –independent effects that are determined by the \(\alpha\) subunit with which it is co-expressed. Also, \(\alpha\) and \(\beta\) subunits appear to modulate Na\textsubscript{v} gating through additive SA-dependent and –independent effects.

Supported by NIH R-01 AR45169
Differential sialylation modulates voltage-gated Na$^+$ channel function throughout the developing myocardium

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Correct gating of voltage-gated sodium channels (Na$_v$) is necessary for maintaining normal heart rhythms. Comparison of Na$_v$ gating in neonatal and adult cardiomyocytes from atria and ventricles indicated that neonatal ventricular Na$_v$ gated following a ~10 mV greater depolarization than did the Na$_v$ expressed in atria or in adult ventricles. Here we questioned whether development- and/or chamber- dependent changes in channel-associated functional sialic acids account for these gating shifts. Thus, desialylation of the neonatal ventricular Na$_v$ had no effect on any aspect of channel gating. However, all gating characteristics for the atrial and adult ventricular Na$_v$ shifted significantly to more depolarized potentials with desialylation. Immunoblot analyses indicated that atrial and adult ventricular Na$_v$ a subunits are more heavily sialylated than the neonatal ventricular a subunit, with approximately 15 fewer sialic acid residues attached to the neonatal ventricular a subunit. The data indicate that differential sialylation of myocyte Na$_v$ a subunits is responsible for the majority of developmental and chamber-specific remodeling of Na$_v$ gating observed here. Thus, a novel mechanism is described by which cardiac action potential initiation and conduction are modulated by the regulated change in channel-associated functional sialic acids.

Supported by NIH-AR-45169.
**N-glycan function in worm and fly development**

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**Abstract:**
UDP-GlcNAc:a3-D-mannoside b1,2-N-acetylglicosaminyltransferase I (GnT I) controls the synthesis of hybrid, complex and paucimannose N-glycans. Mice make hybrid and complex but little or no paucimannose N-glycans. *Drosophila melanogaster* and *Caenorhabditis elegans* make paucimannose but little or no hybrid nor complex N-glycans. GnT I null mice die at embryonic stage E9.5 days. *D. melanogaster* adults with null mutations in the GnT I gene (*dGnTI*) are viable but die shortly after emerging from the puppae. *C. elegans* adults with null mutations in all three worm GnT I genes (*gly-12*, *gly-13*, *gly-14*) (Zhu S *et al*. Biochem.J. 2004, 382: 995-1001) are viable with a normal phenotype and life span under standard laboratory conditions. TKO worm extracts have no detectable GnT I enzyme activity and cannot make 31 paucimannose, complex and fucosylated oligomannose N-glycans present in the wild type worm. The mutant worm has increased amounts of non-fucosylated oligomannose N-glycan structures, a finding consistent with the site of GnT I action. Five fucosylated oligomannose N-glycan structures were observed in TKO but not wild type worms indicating the presence of unusual GnT I-independent fucosyltransferases. Wild type *C. elegans* therefore makes a large number of GnT I-dependent N-glycans that are not essential for normal worm development under laboratory conditions. However, survival of the TKO worm is compromised if the worm is exposed to environmental stress. The data indicate that paucimannose N-glycans are not essential for invertebrate development but play a role in survival on exposure to environmental stress, whereas hybrid and/or complex N-glycans are essential for vertebrate development. The development of GnT I null flies is under study to provide further evidence on this hypothesis. Support by the Canadian Institutes of Health Research (CIHR) and the Canadian Protein Engineering Network Centre of Excellence (PENCE).
Processing of Cell Wall GPI Proteins

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Abstract

In *Saccharomyces cerevisiae* GPI-anchored cell wall mannoproteins are secreted to the cell surface through vesicular transport. At the cell surface the GPI anchor is trimmed and transglycosylated to form a covalent cross-link to beta-1,6 glucan. Among mutants, temperature sensitive for growth and ability to cross-link alpha-agglutinin to the cell wall, one strain, AC59, was complemented by BET1. *BET1* encodes a v-SNARE essential for vesicular transport between the ER and the Golgi. Mutations in *BET1* caused aberrations in cell wall structure including sensitivity to lysis with Zymolyase and hypersensitivity to calcofluor white. In *bet1* mutants secretion of invertase and other proteins is blocked at restrictive temperature (Ferro-Novick), but alpha-agglutinin was excreted into the medium. This secretion was due to continued export of a significant amount of alpha-agglutinin found in intracellular compartments that were after the *BET1*-dependent secretion step. In *bet1* cells, the block in secretion pathway prevented cell wall anchorage of alpha-agglutinin. Thus, a mutation early in the secretion pathway caused aberrant cell wall synthesis, possibly by preventing localization of a key component in wall cross-links.

Supported by NIGMS-SCORE
GPI-mannoproteins play an essential structural role in the cell wall of the budding yeast Saccharomyces cerevisiae. In addition to being involved in cell-cell contact during mating, GPI-mannoproteins form a protective layer that surrounds the outermost surface of the cell. The presence of a GPI anchor, which is post-translationally added to the carboxyl end of the protein, enables attachment of the protein to the cell wall.

In order to determine the cell surface localization kinetics of a GPI-mannoprotein under regulation of the GAL1 promoter, the carboxyl end of the GPI-mannoprotein _α-agglutinin was C-terminally fused to GFP. GFP has been previously used to tag yeast cell wall GPI-mannoproteins, and successful cell wall localization of such proteins has been reported. For similar proteins lacking the cell wall targeting signal (GPI anchor), excretion of the protein into the cell’s growth media has been observed. Estimates for the number of GFP-mannoprotein molecules on the cell surface range in the scale of 10^4-10^9 molecules per cell for GAL1-regulated proteins. However, the time that it takes the cell to synthesize, process, and localize such proteins to the cell wall is unknown. Here, we report cell surface localization kinetics for a GFP-GPI protein in various S. cerevisiae strains, some of which exhibit deficient repression of the GAL1 promoter at various degrees when grown in the presence of glucose (a strong repressor of GAL1). Interestingly, in a strain exhibiting constitutive GAL1 activation (YM4282), kinetics of cell surface localization of the GFP-fusion protein mimics the observed for _α-agglutinin under its endogenous promoter. A similar GFP-fusion protein lacking a GPI anchor was first detected in supernatants from YM4281 and YM4282 cells at 3 hrs and 9 hrs respectively, although quantitation was variable.

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Supported by NIGMS SCORE and SBIR (Mycologics, Inc.)
Sialate-O-Acetylerases: Tools to study the distribution and function of O-acetylated sialic acids

Applied BioTechnology and University of Salzburg, Austria

Applied BioTechnology is a start-up company dedicated to serve the scientific community in the field of Glycobiology. Currently, we supply on a collaborative basis sialic acid-modifying enzymes, which are useful to identify specific modifications of sialic acids. Our product line currently includes recombinant esterases specific for either 9- or 4-O-acetylated sialic acids. In the near future, a torovirus esterase specific for double-O-acetylated sialic acids will be made available. Also in the pipeline are recombinant viral lectins which bind to different forms of sialic acids. In the past, we have expressed recombinant esterases and used them as reference to identify the receptors for viral pathogens infecting life stock and humans.
**Solid-phase Synthesis of mucin-like glycopeptides**

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**Abstract** Tₐₐₐₐₐ Nₐₐₐₐ antigen building blocks N-((9-fluorenylmethoxycarbonyl)-O-(3,4,6-tri-O-acetyl-2-azido-2-deoxy-D-galactopyranosyl)-L-serine/L-threonine pentafluorophenyl ester [Fmoc-L-Ser/L-Thr(Ac₃-D-GalN₃)-OPfp, 1/2] have been synthesized by two different routes, which have been compared. Overall isolated yields [three or four chemical steps, and minimal intermediary purification steps] of enantiopure 1 and 2 were 5–18% and 6–10% respectively, based on 3,4,6-tri-O-acetyl-D-galactal. Intermediates 3,4,6-tri-O-acetyl-2-azido-2-deoxy-D-galactopyranosyl bromide and 3,4,6-tri-O-acetyl-2-azido-2-deoxy-D-galactopyranosyl chloride were used to glycosylate N-((9-fluorenylmethoxycarbonyl)-L-serine/L-threonine pentafluorophenyl esters [Fmoc-L-Ser/L-Thr-OPfp,]. Previously undescribed low-level dehydration side reactions were observed at this stage; the unwanted by-products were easily removed by column chromatography.

Using these building blocks, the fully glycosylated glycopeptide Ac-Pro-Thr(_D-GalNAc)-Thr(_D-GalNAc)-Thr(_D-GalNAc)-Pro-Leu-Lys-NH₂, which features three consecutive O-glycosylated Thr residues and mimics a portion of mucin 2, has been prepared by solid-phase synthesis. Seven partially glycosylated peptides based on the same amino acid sequence were synthesized as well. This suite of molecules allowed a systematic analysis of synthetic protocols. N-((9-fluorenylmethoxycarbonyl)-O-(3,4,6-tri-O-acetyl-2-azido-2-deoxy-D-galactopyranosyl)-L-threonine pentafluorophenyl ester [Fmoc-L-Thr(Ac₃-D-GalN₃)-OPfp] was used as a building block that coupled efficiently when used in a relatively low molar excess, i.e., ~1.5 equiv, with N,N-dimethylformamide (DMF) as the solvent. For conversion of the azido group to the N-acetyl function, direct treatment with thioacetic acid was preferred over a two-step procedure involving reduction with dithiothreitol (DTT) followed by N-acetylation. Effective O-deacetylation in solution, was achieved by treatment with sodium methoxide (10–15 mM; ~5 equiv) in methanol. On-resin deacetylation techniques were also examined, using sodium methoxide (6–10 mM) in DMF/methanol (17:3) or hydrazine (70 mM) in methanol. The more convenient on-resin technique in DMF/methanol gave yields similar to solution conditions, and promises to be widely useful for solid-phase glycopeptide synthesis. HPLC profiles showed that free glycopeptides elute earlier than the corresponding O-acetylated derivatives, and that retention times vary systematically with the number of sugar moieties. ¹H NMR studies carried out in water showed an increase in conformational organization of glycopeptides with increased density of glycosylation.
Conformational aspects of GalNAc transferase glycopeptide substrates

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Abstract - Structural characteristics of a series of glycopeptides based on a MUC2-like primary peptide sequence PTTTPLK are being investigated using solution NMR methods. The glycopeptides have various degrees and permutations of GalNAc glycosylation on the T residues and have been prepared by solid-phase synthesis methods. Some of these glycopeptides have previously been examined by others as substrates for several GalNAc transferases, (1) thus our studies can provide a context for understanding their structure-function relationships and factors contributing to the ultimate pattern of O-linked glycosylation. These studies further can provide insights into the onset of conformational change as a function of the density of glycosylation which can help elucidate the impact of incomplete glycosylation on the overall mucin glycoprotein organization. The NMR data show that conformational order is found to increase with the degree of glycosylation. Interestingly, the spectral fingerprint found for the peptide backbone when all three threonine residues are glycosylated is quite similar to that found for the case of the S*T*T* sequence and an S*S*S* sequence we have also studied, (2) indicating that the glycosylation rather than the choice of S or T residues dominates the molecular organization and the orientation of the sugar residues. In reported studies of antibodies arising from vaccination with anti-Tn-glycopeptide anti-tumor constructs, antibody cross-reactivity among Tn glycosylated triplet constructs with various T and S substitutions have been observed, consistent with this conformational analysis. NMR studies of constructs using amino acid analogs based on glycosylated hydroxynorleucine do not indicate peptide backbone ordering. This is also consistent with the hypothesis that proximity of the GalNAc to the peptide backbone is crucial for the structural interactions.

Gene expression analysis of glycosylation-related genes by real-time PCR

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Abstract
Glycan molecules covalently linked to proteins or lipids control vital properties of cells, such as signaling, adherence and migration through the body. The biosynthesis of such glycans depends on the concerted action of many ER- and Golgi enzymes, a process that is tightly ordered and regulated. To understand the function of glycoconjugates in cellular interactions, it is crucial to investigate the regulation of expression of the genes encoding the “glycosylation-related” genes, encompassing large families of glycosyltransferases, glycosidases, and sulfotransferases. We here describe an easy, flexible and reliable method of quantitative real-time PCR to measure the expression levels of more than 80 human glycosylation-related genes that mainly encode common enzymes involved in and N- and O-linked protein glycosylation and/or glycolipids. Designing and including additional primer sets to detect more genes can easily extend the system. To allow the normalization of gene expression data obtained by real-time PCR within different cells, tissues or under different experimental conditions, a protocol is provided to detect genes suitable for use as endogenous reference genes (1). Several examples for application of the real-time PCR method, such as in the analysis of the regulation of glycosyltransferase expression in HUVEC by TNFα, are shown (2,3).

Core 2 $\beta$1-6 N-acetylglucosaminyltransferase-III selectively contributes to P-selectin ligand formation in activated CD8 T cells

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Abstract

C2GlcNAcT-I gene-deletion studies have demonstrated an essential role for this enzyme in the control of selectin ligand mediated cell trafficking, while little is known about the role of the two alternate core 2 isoenzymes: C2GlcNAcT-II and C2GlcNAcT-III. We have examined activated splenocytes from C2GlcNAcT-Inull mice for their ability to express P-selectin ligands (P-sell) and have found that C2GlcNAcT-I independent P-sell formation can occur in CD8 T cells, but not in CD4 T cells, activated under high density cell culture conditions. Cell surface binding of P-selectin could be inhibited with the neutralizing anti-PSGL-1 antibody, 2PH-1, confirming PSGL-1 as the P-selectin ligand. CD8 T cells from C2GlcNAcT-Inull were capable of rolling under shear flow on immobilized P-selectin. Real time RT-PCR analysis identified significant levels of C2GlcNAcT-III, but not C2GlcNAcT-II, RNA in activated splenocytes, identifying the C2GlcNAcT-III enzyme as the likely source of core 2 activity. Up-regulation of P-sell correlated with an increase in core 2 enzyme activity measured by a standard enzymatic assay and by cell surface binding of the core 2 sensitive mAb 1B11, revealing the well established C2GlcNAcT-I substrates CD43 and CD45 as further physiological targets of C2GlcNAcT-III. To assess whether C2GlcNAcT-I independent P-sell induction occurs under in vivo conditions, we analyzed T cells from mice transgenic for the male antigen (HY) T cell receptor. CFSE labeled HYtg CD8 T cells from female C2GlcNAcT-Inull or wild type donors were transferred into male recipient mice. The CD8 T cell response along with P-sell induction was measured 2-4 days after cell transfer. Both wt and C2GlcNAcT-Inull CD8 T cells showed a comparable proliferative response. While wt controls expressed high levels of P-sell, C2GlcNAcT-Inull cells also expressed P-sell, although at reduced levels. Our data thus indicate that C2GlcNAcT-III may contribute to P-sell formation and cooperate with C2GlcNAcT-I in the control of CD8 T cell trafficking.
Expression of the L5 carbohydrate epitope during neural differentiation of murine embryonic stem cells and P19 EC cells

Simone Diestel[1], Christine Laurini[1], Rachel Buschwald[2], Andrea Streit[3], Oliver Brüstle[2] and Brigitte Schmitz[1]

Abstract
The L5 carbohydrate epitope has been characterized as a 3´-fucosyl-N-acetyllactosamine (Le³) attached to a biantennary core fucosylated N-glycosidically linked glycan truncated on the 1-3 arm. In the chick embryo it has been shown that L5-positive cells can be neuralized and that in particular L5 is functionally implicated in neural competence, the responsive capacity of tissue receiving neural inducing signals.

During neural induction L5 is expressed on an unknown 220 kDa glycoprotein (L5-220), which is downregulated during neural differentiation. Later in development L5 is associated with a 450 kDa protein. Only L5-220 can be upregulated by HGF/SF. Furthermore HGF/SF maintains L5-220 expression and neural competence implicating an important role of L5-220 in neural induction.

As in the chick, L5 is expressed in the mouse embryo during the stage of neural induction. To study the regulated L5 expression and to identify the 220 kDa protein we used murine P19 embryonal carcinoma (EC) cells as well as murine embryonic stem (ES) cells, which can be differentiated in vitro into neural cells. Results obtained from indirect immunofluorescence analysis indicate that L5 shows a similar expression during murine ES and P19 EC cell differentiation into neural cells as chick embryos. In order to obtain insight into regulatory mechanisms determining the expression of L5, microarray analyses using the Glyco-gene Chip array are underway.
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