Structural analysis of cytokine signaling modulators

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Abstract
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A MALDI-TOF analysis was employed to identify the N-glycans involved in modulating signal transduction in wild type and null Mgat 5 cells grown with different GlcNAc supplements. Native N-glycan profiling showed alterations in N-glycosylation generated by GlcNAc supplements through stimulation of hexosamine pathway. After methylation the tetra-antennary and poly-N-acetyl lactosaminylated glycans were identified only in Mgat 5 wild type cells either supplemented or not with GlcNAc. These structures were absent from the signaling deficient Mgat 5 null cells. Although lattice formation and tumor progression are reestablished after GlcNAc feeding, the preferred galectin-3 ligands were absent from Mgat 5 null cells. Collision Induced Dissociation in a MALDI-IT-TOF instrument was used for N-glycan structural determination. For monitoring the alterations in N-glycosylation, null and wild type Mgat 5 cells were grown in medium supplemented with increasing GlcNAc concentrations. The N-glycans were extracted and subjected to neuraminic acid release to facilitate quantitative estimation. Oligosaccharide profiles for the Mgat 5 wild type cells showed little variation in the high mannose glycan series and a slight increase in the relative abundances of complex type glycans. In the Mgat 5 null cells the increments in GlcNAc concentration determine increasing levels of bi- and triantennary glycans and decreasing levels of high mannose glycans. These suggest that present in a higher number the glycans with lower LacNAc multiplicity are able to establish lattice interactions with adequate avidity to stimulate cytokine signaling and tumor progression in a Mgat 5 null background.

Keywords
Biology, Genetics, Chemistry, Biochemistry

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STRUCTURAL ANALYSIS OF CYTOKINE SIGNALING MODULATORS

BY

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B.S., University of Bucharest – Bucharest, Romania, 1998

DISSERTATION

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in
Genetics

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ABSTRACT

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University of New Hampshire, May, 2006

The N-linked glycans attached to cytokine receptors modulate signal transduction by interacting with galectin-3 and generating a cell surface lattice. This opposes constitutive endocytosis and reduces the thresholds for cytokine signaling. The preferred ligands for galectin-3 are the poly-N-acetyl lactosaminylated tetra-antennary glycans which are synthesized by Mgat 5 and are present at high levels in tumor cells. A null mutation in Mgat 5 inhibits lattice formation and cancer progression in cells with an oncogenic background. GlcNAc feeding reestablishes lattice formation, cytokine signaling and tumorigenesis.

A MALDI-TOF analysis was employed to identify the N-glycans involved in modulating signal transduction in wild type and null Mgat 5 cells grown with different GlcNAc supplements. Native N-glycan profiling showed alterations in N-glycosylation generated by GlcNAc supplements through stimulation of hexosamine pathway. After methylation the tetra-antennary and poly-N-acetyl lactosaminylated glycans were identified only in Mgat 5 wild type cells either...
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CHAPTER 1

BACKGROUND AND HYPOTHESIS

The multiple and diverse functions that cells perform during their lifetime are regulated by the cell’s environment through signal transduction. This communication system involves an environmental signal, a cellular signal receptor and an intricate sequence of intracellular biochemical reactions. The signal is amplified by the enzymatic cascades downstream receptor and activates specific transcription factors to produce complex metabolic responses with implications in cell nutrition, respiration, proliferation, differentiation and cell cycle control. Herein, I am analyzing the biochemical mechanisms and the molecular structures which determine the residency time of cytokine receptors at the cell surface and modulate receptor activation and signal transduction.

Cytokines comprise a diverse group of soluble proteins and peptides, which act as humoral regulators at nano- to picomolar concentrations and which modulate the functions of individual cells or tissues. Although they were characterized primarily as factors involved in activation and proliferation of immune cells, later studies have shown that they are able to influence growth and differentiation of a large array of cell types which present a surface receptor. Different cytokines and growth factors are produced by a wide range of cell types
and can exert their biological function on targets situated adjacent or remote from
the secretory cell. Although most cytokines have specific receptors and defined
functions they also show redundancy by overlapping their interactions and
generating a network of interdependent pleiotropic effects (Olsson 1993).

Cytokine synthesis is initiated by activation factors or by other cytokines
and is regulated, depending on cell type and developmental age, through gene
expression, alternative mRNA splicing and various posttranslational
modifications. Also, many cytokines balance their own concentration by providing
feedback to the biosynthetic pathways through an autocrine mechanism
(Oppenheim et al. 2000).

Another level of cytokine activity regulation is through expression of their
transmembrane receptors. Ligand availability and the absolute number of
receptors on the cell surface are major limiting factors for signal transduction
(Gurdon and Bourillot 2001). Signaling down-stream of receptor tyrosine kinases
promotes vesicular trafficking and constitutive endocytosis (Nishikawa et al.
1988). Receptor down-regulation through endocytosis is the result of ligand-
induced internalization of receptor/ligand complexes and their subsequent
degradation by lysosomes. The internalized receptor molecules can also be
recycled and used for reassembly of new transmembrane receptors (Sorkin and
Waters 1993). By promoting receptor internalization, endocytosis has an
important role in terminating the cytokine influence on the cell surface. An up-
regulated signal from the cytokine molecules or a mechanism that opposes
constitutive endocytosis could account for the elevated number of cytokine
receptors often encountered at the cell surface in carcinomas.

Although tumor progression can be attributed to multiple factors, sustained growth signal transduction has a major role in deciding the cell's fate. Different cytokines can work in parallel to supply the cell with signals for proliferation. At the same time, a specific class of cytokines called "cellular survival factors" generate secondary signals, which prevent apoptosis and act in synergy with the growth factors to maintain colony propagation (Balkwill 2001). Because of these effects, many of the genes that encode different segments in the signal transduction cascade or receptor proteins are considered proto-oncogenes.

Interestingly, most of the cytokines involved in cell proliferation or differentiation, as well as their receptors, are glycoproteins. During signal transduction, cytokines that possess lectin domains can enhance multimerization through interactions with the carbohydrates on another monomer or with carbohydrate structures on receptors or matrix molecules (Opdenakker et al. 1995). At the same time, the complex sugars expressed on the extracellular segment of the cytokine receptors can establish multivalent interactions with cell surface lectins and generate a local superstructure which maintains signal transduction across the membrane. The fact that complex-type branched glycans and cytokine receptors are commonly up-regulated in tumors, which correlates with poor prognosis (Fernandes et al. 1991; Seelentag et al. 1998), suggests their role in modulating cell fate through the interactions they establish at the cell surface.
1.1 The Hexosamine Pathway Supplies the Building Blocks for N-glycan Biosynthesis

Glucose is the universal nutrient preferred by most organisms as the primary source of carbon and energy, with fundamental roles in cell growth and proliferation. This may explain why cells have developed numerous and sophisticated glucose-sensing mechanisms.

Single celled eukaryotes perceive glucose as a nutrient, but also as an anabolic signal which binds and activates an array of receptors. These signals influence cell polarity and migration towards the nutrient, glucose uptake by activation of membrane transport and glycolysis with production of energy and cell growth (Kaniak et al. 2004). To modulate cellular morphogenesis and proliferation, multicellular organisms have developed intricate mechanisms which rely on a complex network of information received from cooperating or antagonizing cytokines (Olsson 1993). In this way, the cells' activities can be finely tuned to their own needs, but also to the requirements of their systemic environment. Although glucose remains the preferred nutrient in animals, the signaling process is dominated by cell-cell contacts and cytokines influences (Ozcan and Johnston 1999). Glucose availability and its commitment to glycolysis is required for signaling downstream of anabolic receptor tyrosine kinases. This provides positive feedback to membrane transporters and stimulates glucose up-take and glycolysis in cultured mammalian cells (Gottlob et al. 2001). At the same time, glucose gives positive feedback to cytokine signaling
pathways and maintains receptor activation (Overturf et al. 1999). This project identifies the dependency between nutrient availability and regulation of growth and proliferation signal transduction in tumor cells.

Most mammalian cells have developed membrane transporters specialized for unique monosaccharides and capable of scavenging the environment for traces of nutrient (Panneerselvam and Freeze 1996). Although these transporters can be very efficient, the highest monosaccharide traffic is attributed to glucose and fructose. Glucose is used in a series of interdependent metabolic reactions to generate almost any other type of monosaccharide (Figure 1). After phosphorylation by hexokinases and isomerization, monosaccharides are activated through a reaction of nucleotide transfer. The high-energy donors generated in this way are ready to be used in glycoconjugate biosynthesis. Although many monosaccharides are interconvertible by establishing equilibrium reactions between each other, the biosynthetic pathways can be tightly regulated by the ratio between the quantities of available monosaccharide and those needed in construction of various glycoconjugates. The surplus or deficiency of certain monosaccharides provides feedback to the enzymes that catalyze the rate-limiting transformations in the pathway. Another control level of monosaccharide biosynthesis is through the energy-consuming reactions, which are dependent on glycolysis and the cell's energy charge. These observations suggest that nutrient availability influences not only glycolysis, but also monosaccharide interconversion and synthesis of glycosylated molecules.
Transformation of glucose to glucose-6-phosphate is followed by isomerization to fructose-6-phosphate until equilibrium is established. These reactions bring the metabolic pathway to a dichotomy because fructose-6-phosphate is the common entry point for both glycolysis and the hexosamine pathway (Bendiak and Schachter 1987). If phosphofructokinase is activated, the monosaccharides enter glycolysis to provide the cell with energy. On the other hand, activation of glutamine-fructose-6-phosphate aminotransferase (GFAT) leads to \textit{de novo} biosynthesis of uridine diphosphate N-acetylglucosamine (UDP-...
GlcNAc) through the hexosamine pathway. The reactions catalyzed by these two enzymes are unidirectional and commit the substrate to irreversibly follow one pathway or the other.

Interestingly, overexpression of GFAT in skeletal muscle of transgenic mice results in severe insulin resistance (Hebert et al. 1996). Also, shunting the reaction catalyzed by GFAT through infusion of glucosamine directly activates the hexosamine pathway and generates insulin resistance in skeletal muscle of normal rats (Giaccari 1995; Rossetti et al. 1995). Insulin resistance is a cell saturation signal, which implies that the hexosamine pathway plays the role of a nutrient sensor. This suggests that the two competing pathways, hexosamine synthesis and glycolysis, have important roles in maintaining metabolic homeostasis by integrating information received from anabolic receptors.

1.2 UDP-GlcNAc Is Rate Limiting for Branching of Complex-type N-linked Glycans

The primary effect of hexosamine pathway stimulation by nutrient availability is an increase in the intracellular concentration of N-acetylglucosamine (GlcNAc) which is followed by transformation to UDP-GlcNAc. This is a high-energy molecule which can be used for generating other N-acetylhexosamines (HexNAc) or for biosynthesis of various glycoconjugates (Figure 1). N-linked glycosylation is one of the major metabolic pathways that use UDP-GlcNAc for synthesis of a large repertoire of structurally diverse asparagine
(N) - linked glycans.

N-linked glycosylation is a co-translational modification of nascent proteins which takes place in the lumen of the rough endoplasmic reticulum (ER). This process is regulated contextually, depending on the cells’ developmental stage and the specific functions performed during that time. Because of this, N-glycosylation shows micro-heterogeneity, which means that proteins with identical amino acid sequence can have different glycosylation sites occupied and that each occupied consensus site can carry different glycan structures (Matthijs et al. 1998). Although glycosylation defects provide insight into its physiological functions, at times these are difficult to understand due to their pleiotropic effects or their various degrees of penetrance, imposed by micro-heterogeneity.

N-linked glycosylation plays important roles in different physiological aspects like cell adhesion and mobility, cell-cell interactions, cell differentiation or proliferation, signal transduction and tissue morphogenesis. All these processes have major implications in deciding the cell’s fate during embryogenesis (Armant et al. 1986; Campbell et al. 1995), ontogeny (Cremer et al. 1994) or tissue regeneration after injuries. Alteration of N-linked glycosylation leads to aberrant signal transduction, loss of cell cycle control, cell proliferation, tumor progression and metastasis, impaired differentiation and development.

Moreover, N-glycosylation modulates the immune system through the interactions established between the host and parasite. Antigen – antibody coupling, self vs. foreign recognition, leukocyte proliferation (Tulp et al. 1986) and
migration into infected tissues (Hanasaki et al. 1994) and cytokine signaling through lectin-ligand interactions are all processes mediated by N-linked glycans or other glycoconjugates.

N-linked glycan biosynthesis starts in the cytosol by activation of GlcNAc-1-phosphotransferase, which adds one UDP-GlcNAc molecule to dolichol phosphate lipid inserted in the endoplasmic reticulum membrane (Figure 2). This is followed quickly by addition of a GlcNAc molecule and five mannose residues, in a sequential and linkage specific manner. At this point the dolichol phosphate/oligosaccharide complex flips from the external ER membrane side to the internal side and becomes available to the lumenal enzymes. (Rosner et al. 1982).

![Figure 2. Biosynthesis of lipid linked glycans. Adapted from Varki et al. 1999.](image-url)
The biosynthesis continues with sequential attachment of four additional mannosates to the growing lipid-linked oligosaccharides, followed by transfer of three glucose residues. Production of the final lipid-linked oligosaccharide GlcNAc$_2$Man$_9$Glc$_3$, as well as the intermediates, is a highly conserved process in eukaryotes and some prokaryotes (Trombetta 2003) and it serves as the precursor for N-linked glycan synthesis.

During or after translation, certain proteins enter into the ER for further processing. The GlcNAc$_2$Man$_9$Glc$_3$ moiety is transferred en block by the oligosaccharyltransferase complex from the dolichol-phosphate to the nascent proteins and facilitates correct folding of glycoproteins (Hammond et al. 1994). The glycans are linked to proteins by the asparagine acceptor in a minimal required sequence of Asn-Xaa-Thr/Ser, where Xaa can be any amino acid except for proline (N-X-T/S sites).

Attachment of oligosaccharides to polypeptides is essential in eukaryotes. Although some glycoproteins are functional when their N-glycans are removed enzymatically or by site-directed mutagenesis, total absence of N-glycosylation is lethal, as shown by mutant cells or by cells treated with tunicamycin, an N-glycosylation inhibitor (Elbein 1987; Kukuruzinska et al. 1987).

Once attached to proteins, N-linked glycans undergo a reverse processing facilitated by a series of glycosidases residing in ER and Golgi. During glycoprotein folding, the three terminal glucose residues are released sequentially. This allows mannosidases to remove the unsubstituted mannose.
monomers in a stepwise fashion and to generate a series of high-mannose type glycans.

If the chains are trimmed down to only three or five mannose residues, the oligosaccharides serve as substrates for N-acetylglucosaminyl (GlcNAc) transferases. These Golgi-located enzymes use the UDP-GlcNAc donors, provided by hexosamine pathway, to generate hybrid and complex-type N-linked glycans.

Interestingly, the quantity of UDP-GlcNAc produced and transported to Golgi is rate limiting for GlcNAc transferases and implicitly for synthesis of complex-type N-glycans (Oguri et al. 1997; Van den Eijnden et al. 1988). The large quantity of nutrient required and absorbed by anabolic cells activates glycolysis, which supplies the cell with energy and also stimulates the hexosamine pathway, which produces UDP-GlcNAc. These observations suggest that active anabolic cells generate a larger pool of complex type glycans than other kinds of functional cells and explain, in part, context-dependent glycan microheterogeneity.

At the same time, tissue cultures set with low glucose concentrations in the growth medium reduce the efficiency of epidermal growth factor receptor (EGFR) substitution by the dolichol-phosphate oligosaccharyltransferase complex (Konishi and Berk 2003). Severe nutrient shortage inhibits cell growth and proliferation and also impairs substitution of N-X-T/S sites.

A bioinformatics study performed by our collaborators shows that cytokine receptors which stimulate cell proliferation, growth and oncogenesis have longer
extracellular domains and higher incidence of glycosylation sites than the receptors involved in morphogenesis (Lau et al. in prep.). These findings suggest that signal transduction can be modulated in proliferative cells through expression on the surface of a larger or more diverse population of complex-type N-glycans generated by activation of the hexosamine pathway and nutrient availability.

1.3 Complex-type N-linked Glycan Diversification

by GlcNAc Transferases

N-linked glycan processing in the Golgi is controlled by genetic, epigenetic and environmental factors. The structural variability of complex-type glycans depends on cell-specific regulation of glycosyltransferase genes, concentration of monosaccharide donors and competition between enzymes for acceptor intermediates as well as the stereochemical environment created by the protein sequence around the glycosylation site (Dennis et al. 1999).

As the glycoproteins pass through the Golgi compartments, the attached oligosaccharides undergo remodeling by different families of glycosyltransferases. GlcNAc transferases (T1-T6; Figure 3) perform their biological activities in medial and trans Golgi and generate a pool of complex-type glycans with various contents of GlcNAc residues. At the same time, this class of enzymes is responsible for branching the N-linked glycans and producing precursors for bi-, tri-, tetra-, and penta-antennary structures.
Figure 3. Golgi N-linked glycan processing. Adapted from Dennis et al. 1999.
GlcNAc transferase I (GnTI or TI) adds the first GlcNAc residue to the three or five mannose core in the position $\beta-1,2$ of the mannose residue linked in $\alpha-1,3$ to the middle mannose (Figure 4). This derivatization is required for subsequent modifications by other transferases. In the animal kingdom GnTI function has proven to be essential for organogenesis because complete absence of its products, generated by gene knockout, is lethal in embryos (Ioffe and Stanley 1994; Metzler et al. 1994).

Figure 4. N-glycan branching by GlcNAc transferases.
Adapted from Varki et al. 1999.

GnTII (TIi) adds the second GlcNAc residue to the tri-mannosyl core in the position $\beta-1,2$ of the $\alpha-1,6$ mannose (Figure 4) and generates the precursor to biantennary structures. GnTII processing is required for branching of complex N-glycans by other transferases. Although GnTII mutant embryos survive, the
impairment of the enzyme activity often becomes lethal after birth due to multiple organ and system failures (Jaeken et al. 1994; Chui et al. 1997).

Activation of either GnTIV or GnTV produces precursors to the triantennary structures by addition of GlcNAc residues to the core α 1-3 mannose and α 1-6 mannose, respectively. Although the two enzymes compete for the same substrate, derivatization by GnTV requires GnTII activity and facilitates GnTIV branching. N-glycan processing through activation of both enzymes produces oligosaccharides with four GlcNAc residues, which can be substituted further by galactosyltransferases and sialyltransferases to generate mature tetra-antennary structures (Figure 3).

GlcNAc transferase activities initiate N-glycan diversification by providing substrates for other glycosyltransferases, which generate a plethora of partially or fully decorated oligosaccharides. This extensive structural variability allows glycoproteins to establish multistep interactions with a wide range of lectins situated mostly on the cell surface. The activity of glycosyltransferases takes place in Golgi, an organelle dedicated to production of cell surface and secreted structures and generates the lectin ligands.

Although abolition of the initial steps of Golgi N-glycan remodeling is lethal, null mutations in the genes overseeing later reactions in the pathway generate less severe phenotypes. In Caenorhabditis elegans ablation of the gly-2 gene, which is a homologue of the GnTV gene in mice, is without visible defects despite resulting in an enzymatically null phenotype (Warren et al. 2002). These observations suggest that the upstream and more conserved parts of the N-
glycan remodeling pathway have well rooted implications in modulating cell fate during morphogenesis and development, while the newly evolved structures have roles in finely regulating ultrasensitive interaction in a systemic organization.

1.4 Roles of Mgat 5 in Signal Transduction and Cancer Progression

GnTV or UDP-N-acetylglucosamine: α-1,6 mannoside β-1,6 N-acetylgalactosaminyl transferase is encoded by the Mgat 5 gene in mammals. This enzyme adds a GlcNAc residue in the β-1,6 position to the α-1,6 core mannose and generates half of the tri-antennary glycan pool and all tetra-antennary glycans. Mgat 5-modified glycans are preferred substrates for initiation of poly-N-acetyl-lactosaminylation (Cummings and Kornfeld 1984). Although the tetra-antennary structure is not required for addition of LacNAc or [Galβ1-4GlcNAcβ1-3] extensions, the presence of the core β-1,6 GlcNAc linkage enhances synthesis of poly-LacNAc termini (Yamashita et al. 1984; Pierce and Arango 1986). The length of the polylactosamine extension is influenced by the availability of activated monosaccharides, the concentration of transferase β-1,3 GlcNAc-T(i) and also by the competition among chain terminating enzymes like sialyltransferases or fucosyltransferase. The [Galβ1-4GlcNAcβ1-3] unit can be repeated between 2 and 10 times and it can be attached to all of the branches.
This generates another level of variability among the N-linked glycan structures and facilitates interaction with lectins with different affinities.

Galectins are a family of soluble lectins that bind β-galactosides, but with relatively low affinities. However, the binding affinities increase depending on the stereochemical environment of β-galactosides, especially if this residue is linked into a lactose or LacNAc sequence (Leffler and Barondes 1986). Galectin affinities for N-glycans are proportional to GlcNAc-branching and the number of N-acetyllactosamine units (Hirabayashi et al. 2002). Being the preferred substrate for poly-N-acetyllactosaminylation, the tetra-antennary glycans synthesized by Mgat 5 provide the highest affinity ligands for galectins.

The only antiapoptotic member of the glectin family (Kim et al. 1999), galectin-3 (Gal-3) binds with high affinity to the [Galβ1-4GlcnAcβ1-3] unit through its carbohydrate recognition domain. At the same time, the non-lectin domain of galectin-3 mediates pentamer formation in the presence of multivalent ligands, thereby cross-linking glycoproteins in proportion to ligand concentrations (Ahmad et al. 2003).

Cytokine receptors presenting Mgat 5 modified N-glycans with poly-N-acetyllactosamine extensions interact with galectin-3 and form a molecular superstructure at the cell surface. The resulting lattice of multimeric galectins and receptor glycoproteins opposes constitutive endocytosis. In this way the molecular lattice reduces the thresholds for cytokine mediated signaling by increasing the time of receptor residency at the cell surface (Partridge et al. 2004). The number and types of N-glycans attached to the receptor extra-cellular
domain determines the lattice strength and has a proportional impact on cytokine signaling.

Culturing tumor cells expressing Mgat 5 in the presence of lactose, a competitor for N-linked glycans in binding galectin-3, reduces receptor responsiveness to EGF. Treatment with sucrose, a disaccharide similar to lactose but without galectin-3 ligand properties, had no effect on receptor activation (Partridge et al. 2004). This comparison shows that signal transduction is modulated by the lattice through the N-linked glycans attached to cytokine receptors and not by other possible Mgat 5 modified structures with different cellular localizations.

Mgat 5 expression as well as branched complex glycans are commonly up-regulated in cancer and are required for growth signal transduction, epithelial-mesenchymal transition, cell motility and metastasis. Moreover, Mgat 5 expression correlates well with poor prognosis (Fernandes et al.1991; Seelentag. et al. 1998; Granovsky et al. 2000).

Tumors induced in transgenic mice by polyomavirus middle T oncogene (PyMT) are generated by activation of Src, phosphatidylinositol 3-kinase and Ras pathways and are dependent on cytokine receptor stimulation (Webster et al. 1998). Interestingly, the Mgat 5 null mutation (Mga 5⁻/⁻) is able to decrease receptor sensitivity to cytokines and to suppress oncogenic progression in mammary epithelial tumor cell lines isolated from PyMT transgenic mice (Partridge et al. 2004; Granovsky et al. 2000). Measurements of phosphorylation and nuclear translocation of extracellular signal regulated kinase (Erk) and Sma

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and Mad protein (Smad) transcription factors show a 100-fold decrease of receptor sensitivity to epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin growth factor (IGF-1) and fibroblast growth factor (FGF) and only a 3-fold decrease of receptor sensitivity to transforming growth factor β (TGF-β), respectively (Partridge et al. 2004). The glycoprotein-galectin lattice is deficient in Mga5/− tumor cells, which determines an increased rate of receptor removal from the cell surface. Receptor down-regulation through endocytosis is inversely proportional to the strength of the lattice, which is generated by the number of occupied glycosylation sites per receptor and the proportion of poly-N-acetyl lactosaminylated sugars. The difference in receptor sensitivity to EGF versus TGF-β is determined by the presence of 10 to 12 potentially occupied glycosylation sites on the EGF receptor (Stroop et al. 2000) and only 1 and 3 sites for the two TGF-β receptors.

Transfection of Mga 5/− tumor cells with a retroviral vector carrying the wild type copy of the Mga 5 gene rescues the PyMT oncogenic phenotype and increases signal transduction, suppresses contact inhibition, increases cell motility and metastasis (Partridge et al. 2004). These observations reinforce the role of tetra-antennary and poly-N-acetyllactosaminylated oligosaccharides, synthesized by the glycosylation pathway downstream of Mgat 5, in modulating carcinogenesis through the cell surface multimeric lattice.

Remarkably, GlcNAc supplement to the growth media of Mga 5/− tumor cells reestablishes receptor sensitivity to cytokines and increases cell surface galectin-3 to levels comparable to the ones in Mga 5+/+ (Lau et al. in prep.).
These results suggest that the increased concentrations of absorbed GlcNAc are able to rescue the formation of the receptor glycoprotein-galectin lattice in the absence of Mgat 5 function.

Glutamine-fructose-6-phosphate aminotransferase (GFAT) is the key regulation point of the hexosamine pathway and its activity is tightly controlled through feedback from its product concentration (Sayeski and Kudlow 1996). In Mga5−/− tumor cells GlcNAc supplement bypasses this control point and is able to activate the hexosamine pathway in a low glucose background. Hexosamine pathway excitation generates UDP-GlcNAc, which is used in biosynthesis of various glycoconjugates, among which are the N-linked glycans attached to the extracellular domain of the cytokine receptors.

This project makes an effort to identify the mechanisms by which environmental factors, like nutrient availability or presence of metabolism intermediates, are able to provide temporal adaptations in culture cells. I want to identify the glycan structures produced under Mgat 5 expression and the poly N-acetyl lactosaminylated structures generated by subsequent substitutions.

This is of interest because expression of the Mgat 5 gene is involved in regulation of different physiological processes by generating N-glycan ligands for a variety of functional lectins. Interactions established between galectin 3 and the Mgat 5 modified N-glycans on the T cell receptor (TCR) prevent autoimmune disease by raising the threshold for T cell activation and limiting TCR clustering (Demetriou et al. 2001). Also, in epithelial cancer cells, the presence of Mgat 5 modified glycans facilitates epithelial-mesenchymal transition with loss of
adhesion junctions, reduced contact inhibition and increased cell motility. The highly motile phenotype shown by metastatic cells is characteristic of active leukocytes as well. A null mutation in Mgat 5 suppresses the inflammatory process and inhibits leukocyte extravasation (Partridge et al. 2004).

In our model the Mgat 5 modified glycans increase receptor residency at the cell surface by participating in formation of the glycoprotein - galectin lattice. This stimulates signal transduction and activates Ras, P1 3 kinase and Smad 2 and Smad 3 (Oft et al. 2002). In turn, the Ras-Raf-Ets pathway supplies positive feedback to Mgat 5 expression (Chen et al. 1998).

At the same time, the hexosamine pathway, stimulated by environmental factors, has major roles in maintaining metabolic homeostasis of N-glycosylation by supplying donor molecules for N-glycan biosynthesis. GlcNAc supplements to cell growth media reestablish signal transduction in the Mgat 5 null background.

These findings suggest that both the Mgat 5 modified glycans with poly-LacNAc substitutions and stimulation of the hexosamine pathway are able to generate the cell surface lattice, independent of each other. The objectives of this project are first, to identify the glycan structures involved in formation of cell surface lattice in Mga5+/+ cells and the alterations in N-glycan remodeling produced by GnTV deficit in Mga5+/− background. Second, to identify the modifications of the hexosamine pathway produced by GlcNAc supplements and the effects of these modifications on N-linked glycan biosynthesis. Third, to characterize the glycan structures involved in rescuing the glycoprotein – galectin interactions in the Mga 5−/− cells supplemented with GlcNAc.
CHAPTER 2

EXPERIMENTAL APPROACH

2.1 Propagation of Mgat 5 Tumor Cells

Mammary tumor cell lines were established from spontaneous carcinomas in polyoma middle T (PyMT) transgenic mice on a 129sv x FVB background with either Mgat 5\(^{+/+}\) or Mgat 5\(^{-/-}\) genotypes as previously described (Granovsky et al. 2000). Cell lines Mgat 5\(^{+/+}\) (2.6) or Mgat 5\(^{-/-}\) (2.9) were generated from mammary tumors removed from littermate PyMT transgenic mice.

To supplement the hexosamine pathway with UDP-GlcNAc, cells were grown in glucose-free DME medium (Gibco) supplemented with 0.9 mM D-Glucose and 50 mM GlcNAc (Sigma) with 10% serum for 48 hours. To titrate the effects of GlcNAc, the cells were grown in glucose-free DME media enriched with 0.9 mM D-Glucose and defined GlcNAc supplements. The GlcNAc concentrations in the titration series were: 0 mM, 12.5 mM, 18.75 mM, 25 mM, 31.25 mM, 37.5 mM, 43.75 mM and 50 mM.
2.2 Protein Isolation and Purification

The cells were lysed from frozen with a membrane disruption buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 65 mM dithiothreitol (DTT), 10 mM acrylamide, 40 mM Tris-HCl pH=8 and mammalian protease inhibitor cocktail (Sigma, St. Louis, MO). After 5 cycles of 3 min. each of sonication on ice, the proteins were quantified with the Bradford Assay (Pierce, Rockford, IL) using the photometer mode of a UV-Viz. Spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Total cell lysate proteins were purified by 3 cycles of dialysis: for 2 hours in 20 mM NH₄HCO₃, 0.05% SDS; followed by over night dialysis at 4°C, in 10 mM NH₄HCO₃, 0.02% SDS and then for 2 hours in 5 mM NH₄HCO₃. The dialysis cassettes (Pierce, Rockford, IL) with 7000 MWCO membranes were rehydrated before using. The purified proteins were lyophilized (Freezone 2.5, Labconco, Kansas City, MO) and resuspended in water. For comparative analyses equal quantities of proteins (0.5 mg) were prepared for each cell type and each GlcNAc treatment.

2.3 N-linked Glycan Release

The proteins were denaturated by heating at 100°C for 15 minutes in 0.05 ml 1X denaturing buffer (5% SDS and 10% β-mercaptoethanol). The samples were cooled to room temperature followed by addition of 1/10 volume of 10X G7
buffer (0.5 M sodium phosphate, pH 7.5) and 1/10 volume of 10% NP-40. The
deglycosylation was performed by proteins incubation with 0.05 U of glycerol-free
Peptide: N-Glycosidase F (PNGase F; NEB, Beverly, MA) for 48 hours at 37°C.

**2.4 Protein and N-linked Glycan Separation**

The proteins and detergents from the deglycosylation mixture were
extracted using a previously described method (Reinhold and Reinhold 1999)
with some modifications.

The samples were diluted in 5% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic
acid and passed through C18 SEP-PAK® cartridges (Waters) under gravitational
pressure. The columns were conditioned in advance with three washings of four
ml each of the following aqueous solutions: 1) 80% (v/v) acetonitrile, 0.1% (v/v)
trifluoroacetic acid; 2) 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid; 3) 5%
(v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. After loading the samples the
flow through was collected immediately for retrieving the glycans. The cartridge
was washed with 3 ml of 5% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid
solution and the eluant volumes were pooled together. The oligosaccharide
solutions were evaporated to complete dryness in a SpeedVac® Concentrator
(Savant Instruments, Holbrook, NY).

The proteins were recovered from the C18 cartridge by elution with 50%
(v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. The highly hydrophobic proteins
were eluted with 80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid.
2.5 N-linked Glycan Purification

The N-linked glycans were further purified and desalted using Porous Graphitized Carbon (PGC) columns as described previously (Packer et al. 1998) with modifications.

The powder PGC column bed was washed three times by vortexing in a capped tube with four ml of each of the following aqueous solutions: 1) water; 2) 1 M NaOH; 3) water; 4) 80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid; 5) 25% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid; 6) 25% (v/v) acetonitrile; 7) water. After each wash the tube was centrifuged and the supernatant was removed. The cleaned PGC was packed in a 10 μl pipette tip with an incorporated C18 bed, ZipTip® (Milipore, Danvers, MA). The ZipTip® was fitted to a 1 ml syringe and the mixed column bed was washed with 25% (v/v) acetonitrile solution if the sample contained only neutral glycans or with 25% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid solution if acidic glycans were expected to be present in the sample. For an unknown glycan mixture the column was washed with 25% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid solution. The micro-column was equilibrated with water and the glycan sample, resuspended in water, was passed through the column with a flow rate of 0.2 ml/minute. The flow through was collected and reloaded into the column. The column was washed with water and the flow through was discarded. Neutral glycans were eluted with 25% (v/v) acetonitrile solution, while acidic glycans were eluted with 25% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid solution.
When separation was not desired the glycans were eluted in batch with 25% (v/v) acetonitrile, 0.1% (v/v) trifluor acetic acid solution. The glycan mixtures were frozen immediately after elution and lyophilized.

2.6 Oligosaccharide Reduction

Fresh NaBH₄ solution was prepared by adding 10 mg/ml NaBH₄ in an aqueous solution of 0.01 N NaOH with a final pH between 10 and 12. Dried oligosaccharides were resuspended in the NaBH₄ / NaOH solution, covered with a loose cap and incubated at room temperature for 16 hours. The glycan solution was chilled to 4°C and neutralized with drop-wise addition of 20% acetic acid. After dilution in 2 ml of ethanol, the solution was dried using a nitrogen evaporator N-EVAP™ 112 (Organomation Associates, Berlin, MA).

The excess borate was eliminated by successive evaporations with 2 ml of each of the following solutions: 1) three times with 1% acetic acid in methanol; 2) toluene; 3) two times with 1% acetic acid in methanol; 4) toluene.

After resuspension in water, the oligosaccharides were desalted by passing through a column packed with the protonated form of DOWEX AG 50W X8-400 cation-exchange resin (Sigma-Aldrich, St. Louis, MO). The resin was conditioned in advance by washing it with an aqueous solution of 20% acetic acid followed by successive water washes. The eluant was collected immediately after loading the oligosaccharide mixture and was evaporated to dryness in the SpeedVac®.
2.7 Oligosaccharide Methylation

Glycan methylation was performed under controlled conditions of humidity following a previously described method (Ciucanu and Kerek 1984; Ciucanu and Costello 2003).

The reduced glycan samples were dried in the SpeedVac® and stored under vacuum atmosphere in a desiccator with P₂O₅ over night. The dry glycan samples were re-suspended in 0.2 mL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) and 1% water. About 0.08 to 0.1 g of NaOH powder was added to the samples which were flushed with argon, capped tightly, covered with parafilm and vortexed for 30 minutes. The powdered NaOH was prepared in advance by grinding the pellets in a mortar and baking the powder at 130°C for 5 hours.

For glycan methylation 0.05 mL of iodomethane (Sigma-Aldrich, St. Louis, MO) was added to the samples, which were then flushed with argon, capped tightly, covered with parafilm and vortexed for about 45 minutes. After adding the iodomethane, the samples were light protected by wrapping the tubes in aluminium foil. The reaction mixture was diluted with 1mL dichloromethane (HPLC grade; EMD-Biosciences, San Diego, CA) and stopped by adding, on ice, 1 mL of 10% acetic acid, aqueous solution. The methylated glycans were extracted in the organic phase and purified by repeated washes with water. After the last water wash, the samples were frozen and the liquid organic phase was transferred to a clean tube and dried in a SpeedVac®. The methylated glycans
were resuspended in methanol (HPLC grade) and were ready for mass spectrometry analysis.

2.8 Neuraminic Acid Release

Mild acidic hydrolysis was employed for non-specific release of neuraminic acid residues. Through this chemical approach neuraminic acid and all its analogs were hydrolyzed independent of the type of glycosidic linkage.

The native, reduced glycans from each cell type and GlcNAc treatment were dissolved in 0.5 mL of 2 M acetic acid aqueous solution (EMD-Biosciences, San Diego, CA) and heated at 80°C for 2 hours. The acetic acid was removed by evaporation. The glycan mixture was suspended in methanol (HPLC grade) and evaporated again. For mass spectrometry analysis, the methylated oligosaccharides were resuspended in methanol (HPLC grade).

2.9 MALDI-TOF Mass Spectrometry

The MALDI-TOF glycan profiles were generated using an Axima Curved Field Reflectron (CFR) mass spectrometer (Kratos-Shimadzu Analytical, Manchester, UK) equipped with a nitrogen laser, which emits in the ultraviolet range (λ =337 nm). The instrument was externally calibrated, for the reflectron mode, using a mixture of peptides and protein standards (bradykinin fragment,
P₁₄R synthetic peptide, ACTH fragment, insulin) covering a mass unit range between m/z 700 and 6000.

For each analysis, 0.8 μL of glycan solution was spotted and mixed with 0.8 μL of matrix on a stainless steel target plate and allowed to dry. Before analysis the sample spots were resuspended in 0.5-μL ethanol and dried again.

All spectra were recorded in reflectron positive-ion mode using as matrix 2,5-dihydroxy benzoic acid (DHB, 10 mg/mL in 50% acetonitrile). The samples were ablated with 60% of the maximum laser power by rastering over the target surface. Mass spectra were acquired by averaging 500 profiles generated by 5 laser shots per profile with the post extraction parameter optimized for native glycans around m/z 3000 and for methylated ones around m/z 4500. Spectra processing was performed with Kratos Launchpad software.

2.10 MALDI-IT-TOF Mass Spectrometry

MALDI-IT-TOF tandem mass spectrometry was performed on the Axima QIT instrument (Kratos-Shimadzu Analytical, Manchester, UK). The instrument was calibrated externally with fullerite in "Mid Mass" mode and with insulin B in the "High Mass" mode. The ion trap was calibrated on a "dirt background" sample for a mass range from m/z 400 to 5000. The samples were spotted on the target plate as previously described. The samples were ionized with 30-40% of the maximum laser power while rastering on the plate surface. The number of profiles accumulated was adjusted as a function of the signal.
intensity and some extended to 7000 profiles per spectrum. The fragmentation patterns in MS^n experiments were generated by collision-induced dissociation (CID) with argon gas. The collision energy in the ion trap was adjusted to facilitate enrichment of the ion desired to be fragmented further in the MS^n+1 step. Spectra processing was performed with Kratos Launchpad software.

2.11 Peak Annotation

Peak annotation was performed initially by using the program GlycoMod Tool, a package under the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB). GlycoMod (http://ca.expasy.org/tools/glycomod/) is a computer algorithm which generates a list of possible oligosaccharide compositions depending on parameters inputted by the user. Also, GlycoMod lists all the hits to GlycoSuite Data Base, which encompasses most of the naturally occurring glycan structures reported in the literature.

For peak annotation the GlycoMod hits to GlycoSuite DB were considered preferentially. To support peak annotations and to resolve between multiple hits to GlycoSuite DB, various oligosaccharides were subjected to structural analysis by molecular disassembly.
CHAPTER 3

NATIVE N-GLYCAN PROFILING

The types of interactions that N-linked glycans establish with different classes of lectins define them as modulators of various cellular processes under physiologic or disease conditions. To characterize the N-linked glycans involved in tumor progression, PyMT mammary tumor cell lines were analyzed in an Mgat 5^+/+ or Mgat 5^−/− genetic background. A mass spectrometry analysis of Mgat 5^+/+ or Mgat 5^−/− cell lines would identify the GnTV modified oligosaccharides and their poly N-acetyl-lactosaminylated analogs. These structures establish the glycoprotein – galectin lattice on the cell surface and lower the threshold for cytokine signaling. It would be expected that the tetra-antennary and the poly-N-acetyl-lactosaminylated sugars be absent from the lattice-deficient Mgat 5^−/− cells.

The alterations in glycosylation pathway were monitored for each genetic background, in cells grown in a medium with either low glucose or low glucose supplemented with GlcNAc. This comparative analysis seeks identification of changes in the hexosamine pathway reflected in the N-glycan biosynthesis. We hypothesized that absence of GnTV function in the Mgat 5^−/− cell line inhibits synthesis of tetra-antennary glycans and lattice formation. At the same time,
stimulating the hexosamine pathway with GlcNAc supplements will create a build-up of the glycans upstream of GnTV and increase synthesis of tri-antennary and bi-antennary poly-N-acetyl-lactosaminylated structures. The N-linked glycans were released from total cell proteins, purified and submitted to mass spectrometry analysis.

3.1 Comparative Analysis of Native N-linked Glycans

Mass spectrometry analysis of oligosaccharides released from biological samples may prove to be challenging due to the low concentration of analyte encountered in the complex mixture of molecules extracted from the total cell lysate. In this case two different methods were employed to release oligosaccharides attached to glycoproteins.

Hydrazinolysis is a non-specific method for releasing N-linked and O-linked glycans (Patel et al. 1993). This chemical release method is advantageous in profiling all types of glycans and especially the ones that are attached to proteins in a stereochemically enclosed glycosylation site. The method’s major disadvantage is that, when a small quantity of glycoprotein is subjected to hydrazinolysis, the reaction generates high background levels, which makes it difficult to identify the low concentration molecules in a sample mixture (results not shown). For this reason the glycans were released using peptide N-glycosidase F (PNGase F), an enzyme that specifically cleaves N-linked glycans (Maley et al. 1989). PNGase F hydrolyzes the linkage between the chitobiose
unit and the peptide to yield the complete sugar moiety and a sugar-free peptide in which asparagine is converted to aspartic acid (Mort and Pierce 1995). This enzyme is extremely versatile and all commonly encountered N-linked sugars are susceptible to this enzyme except those occurring on C- or N-terminal asparagines. Also, the glycan substitution in the position α1-3 of the reducing end GlcNAc hinders the enzyme’s access to the glycosylation site and renders the oligosaccharide resistant to enzymatic treatment (Tretter et al. 1991).

Mass spectrometric profiling of released native N-glycans using MALDI-TOF in the positive ion mode is an initial step for identifying oligosaccharides without derivatization. Both neutral and basic glycans can be detected using this method, because these oligosaccharides ionize by forming positively charged Na⁺ adducts. The acidic glycans ionize by losing one proton from each carboxylic group and forming negatively charged species, which cannot be detected in positive ion mode.

A comparative analysis of the Mgat 5⁺/+ and Mgat 5⁻/⁻ cells and those supplemented with GlcNAc provide the native N-linked oligosaccharides profiles in the figures below. The resultant spectra show the series of high mannose glycans generated by mannosidases located in ER and cis-Golgi. The peaks corresponding to high mannose sugars were marked in all of the spectra with a dot. The complex and hybrid glycans show variations in relative abundance for the different GlcNAc treatments.

The Mgat 5⁺/+ cell line grown in low glucose media presents a profile with series of concentrated high mannose glycans and less abundant complex and
hybrid sugars (Figure 5). The succession of reactions in the glycosylation pathway can be followed in the spectrum starting from the Man9GlcNAc2 structure (m/z 1907.6) and going along the reverse processing of high mannose glycans series, with decrease in molecular weight, down to Man5GlcNAc2 (m/z 1081.7) or Man3GlcNAc2 (m/z 935.7). These two structures are substrates for GnTI, which adds the first GlcNAc residue to the N-linked glycan core mannose. The peaks corresponding to hybrid and complex glycans increase in molecular weight as Golgi transferases add successively GlcNAc residues, followed by addition of galactose, fucose and neuraminic acid residues.

Figure 5. Positive ion MALDI-MS profile of native N-glycans from Mgt 5+/+ cells grown in low glucose medium. • High mannose sugars; • Mannose, • GlcNAc, • Galactose, > Fucose

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Bi-antennary and tri-antennary structures were found in Mgat 5\(^{+/+}\) native glycan mixtures while the tetra-antennary peaks show very low intensity or coincide with predominant high mannose peaks. Although neuraminylated sugars may be present in the mixture, they could not be detected in positive ion mode.

Interestingly, oligosaccharides bearing galactose extensions to the [Gal\(\beta 1-4\)GlcNAc] unit were encountered in the Mgat 5\(^{+/+}\) samples at m/z 1827.6, 1973.6, 2135.5, 2339.4. The Gala1-3Gal structure has been found in mammals except for great apes (Galili et al. 1988) and has been identified as the major antigen responsible for hyperacute rejection in xenotransplantation (Kobayashi and Cooper 1999).

In the glycan profile of Mgat 5\(^{+/+}\) GlcNAc supplemented samples, the high mannose series was still predominant in the spectra but the sugars involved in the initial steps of the \(\alpha\)-mannosidase processing decreased in concentration. At the same time the peaks for complex glycans increase in abundance (Figure 6). The bi-antennary and tri-antennary truncated glycans are the direct products of GlcNAc transferases and the peaks corresponding to these structures are elevated in the Mgat 5\(^{+/+}\) GlcNAc supplemented sample. Also, the galactose substituted bi-antennary and tri-antennary glycans were present in higher concentrations in this treatment (m/z 1487.9, 1666.0, 1690.6, 1812.0, 1852.7, 2016.0, 2031.0, 2177.3).

The oligosaccharides bearing galactose extensions to the [Gal\(\beta 1-4\)GlcNAc] unit were found in lower concentrations in the GlcNAc supplemented cells than in the cells grown in low glucose medium.
These findings show that GlcNAc transferases compete with other transferases to remodel complex N-glycans. Also, the changes in glycan abundance in the two Mgat 5+/+ cell treatments suggest that the stimulation of the hexosamine pathway through GlcNAc supplements can influence N-linked glycan remodeling.

The profile for Mgat 5−/− cells grown in low glucose medium presents a high mannose glycan series similar to the one in Mgat 5+/+ cells (Figure 7). At the same time, the spectrum shows elevated concentrations of the products of GnTI and GnTII enzymes (m/z 1139.5, 1285.5, 1341.5, 1388.7). This implies that in
Mgat 5⁻/⁻ cells the initial steps in N-glycan remodeling were activated although the hexosamine pathway was not stimulated with GlcNAc. These observations are consistent with the experimental measurements of UDP-GlcNAc concentrations performed by our collaborators. Their findings indicate that basal UDP-GlcNAc levels in Mgat 5⁻/⁻ cells grown in low glucose were 3-fold greater than in Mgat 5⁺/+ cells (Lau et al. in prep.). Taken together, these findings suggest that in Mgat 5⁻/⁻ cells the hexosamine pathway is artificially stimulated in the absence of GlcNAc supplements and promotes activation of the initial steps in N-glycan processing.

![Figure 7. Positive ion MALDI-MS profile of native N-glycans from Mgat 5⁻/⁻ cells grown in low glucose medium.](image)

- High mannose sugars
- Mannose, GlcNAc
- Galactose, Fucose
catalyzed by GnTI and GnTII. It appears that the *Mgat 5* deficiency impairs the feedback regulation of the hexosamine pathway and N-glycan processing.

Although the elevated UDP-GlcNAc levels are able to influence the initial steps in the N-glycan remodeling, the synthesis of bi-antennary and tri-antennary mature glycans is maintained at relatively low levels in Mgat 5\(^{-/-}\) (Figure 7). These results suggest that in a low nutrient medium the mutant cells are not capable of overcoming the deficit in the glycosylation pathway.

Analysis of glycans released from Mgat 5\(^{-/-}\) GlcNAc supplemented cells show drastic changes in N-glycan remodeling (Figure 8). This spectrum presents

![Figure 8. Positive ion MALDI-MS profile of native N-glycans from Mgat 5\(^{-/-}\) cells grown in low glucose medium supplemented with GlcNAc. • High mannose sugars; • Mannose, • GlcNAc, • Galactose, • Fucose.](image)

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a total reverse in the proportion between the high mannose and hybrid and complex type N-glycans. While the high mannose glycans decrease to one third in relative abundance, the truncated bi-antennary and tri-antennary glycans become the predominant peaks in the spectrum (m/z 1341.4, 1487.4, 1544.4, 1690.4).

Oligosaccharides carrying four GlcNAc residues (m/z 1893.4, 2055.3, 2218.4) were encountered in Mgat 5\textsuperscript{−/−} GlcNAc supplemented cells in elevated concentrations compared to the other treatments where these structures are present in low concentrations or even absent. I speculate that, due to the GnTV deficit these cells could not synthesize the tetra-antennary truncated glycans unless the excess UDP-GlcNAc is able to overcome the energy activation barrier of the Mgat 5 enzyme. The detailed structure of the oligosaccharides with four GlcNAc substituents attached to the core could not be determined before derivatization by permethylation.

As in the Mgat 5\textsuperscript{+/+} GlcNAc supplemented cells, the oligosaccharides bearing galactose extensions to the [Gal\(\beta\)1-4GlcNAc] unit were found in low concentrations or were absent from this treatment. Their low abundances could suggest that the GlcNAc supplements could determine a preferential capping with neuraminic acid of the non-reducing N-glycan termini.

The truncated structures with two, three and four GlcNAc substituents have the highest relative concentrations in the glycan mixture extracted from these cells and are direct products of GlcNAc transferases. These findings show that the GlcNAc supplements regulate N-linked glycan remodeling through
stimulation of the hexosamine pathway and increased concentrations of UDP-GlcNAc. Even more, the impaired regulatory feedback from Mgat 5 sustains biosynthesis of truncated complex glycans and accumulation of these structures in the cells.

The native N-glycan profiling illustrates the interdependency between the hexosamine pathway and N-linked glycan remodeling, but the detection of mature neuraminylated oligosaccharides was not possible using this method. For identification of high molecular weight glycans and for detailed structural analysis the oligosaccharides from each cell treatment were submitted to methylation.
CHAPTER 4

METHYLATED N-GLYCAN PROFILING

Methylation is used to improve the analytical properties of oligosaccharides because it confers several advantages for the mass spectrometric analysis. Methylation enhances carbohydrate detection by extracting the lipophilic products into non-polar solvents thereby purifying the glycans from polar biological components. Glycan methylation with methyl iodide can be catalyzed by methylsulfenyl carbanion (Hakamori 1964) or by sodium hydroxide (Ciucanu and Kerek 1984). The hydroxyl group base-catalyzed ionization method was used for derivatization of oligosaccharides extracted from the Mgat 5 cells with different GlcNAC treatments. The reaction was carried out under controlled conditions of humidity and increased the molecular weight of oligosaccharides by replacement of all labile protons in hydroxyl, carboxyl, and amide groups with methyl.

Moreover, molecular disassembly of methylated sugars produced by collision induced dissociation (CID) facilitates a comprehensive structural understanding of branching and linkage patterns by resolving between free hydroxyl groups and those involved in glycosydic bonds (Geyer and Geyer 1994). By transforming the negatively charged carboxyl groups into esters,
methylation allows detection of acidic glycans through ionization as Na+ adducts in positive ion mode. In this study, mass spectrometric analysis of native glycans showed variations in oligosaccharide processing among the four GlcNAc treatments and Mgat 5 genetic backgrounds and demonstrated a dependency between glycosylation and the hexosamine pathway. Methylation was employed to facilitate detection of high molecular weight oligosaccharides and the ones substituted with acidic monosaccharides. Also, it was used for monitoring the effects of the Mgat 5 null mutation on N-glycans remodeling.

4.1 Comparative Analysis of Methylated N-linked Glycans

An initial hypothesis takes in consideration the important role of poly-N-acetyl-lactosaminylated glycans in establishing the cell surface lattice. This hypothesis proposes that the tetra-antennary structures cannot be synthesized in the Mgat 5/− cells and that the signal transduction in Mgat 5/− GlcNAc supplemented cell line was rescued by potentially poly-N-acetyl-lactosaminylated bi-antennary and tri-antennary sugars. The mass spectrometric analysis of methylated oligosaccharides seeks identification of those component structures needed to re-establish the receptor-galectin lattice.

Analysis of methylated N-glycans from the Mgat 5+/+ cells grown in low glucose medium shows a profile predominated by the high mannose glycan peaks with an averaged relative abundance of approximately 75% of the maximum intensity (Figure 9, Appendix A). Also, the spectrum presents a
relatively high abundance (45% of max. int.) for the pool of bi-antennary glycans substituted with galactose and neuraminic acid (m/z 2085.5, 2261.5, 2465.3, 2495.2, 2669.5, 2809.5, 2826.4, 2983.4). The tri-antennary glycans (m/z 3071.3, 3228.2, 3275.2, 3619.0, 3635.9, 3793.2, 3979.7) are present in relatively low concentrations, approximately 10% of the maximum intensity. The tetra-antennary structures show an even lower relative concentration (4% of max.int.) and present variations in the degree of neuraminylation (m/z 4086.3, 4242.5, 4288.8, 4446.2, 4602.6). Although the averaged relative abundance of poly-N-acetyl-lactosaminylated sugars is less than 1% of maximum intensity, these structures present similar variations in the galactosylation and neuraminylation patterns as the bi-, tri- and tetra-antennary glycans. The panel in the upper right corner of Figure 9 shows an expanded view of the mass range between m/z 3600 and 6000. Over this region the poly-LacNAc oligosaccharides show an increase in molecular weight starting from m/z 4737.0 and they could be detected up to m/z 7000.

In Figure 9 only one possible structure is associated with each oligosaccharide peaks. However, the LacNAc or galactose extensions could be attached in different positions than the ones depicted here. Also, some of the peaks could be generated by ionization of compounds with identical molecular weights but different structures. Many of these oligosaccharides were analyzed by molecular disassembly and the structural details are presented below. Even though most of the N-glycans in Mgat 5 cell lines could be identified, branch
Figure 9. Positive ion MALDI-MS profile of methylated N-glycans from Mgt5+/+ cells grown in low glucose medium
• High mannose sugars; • Mannose, • GlcNAc, ▲ Galactose, ▶ Fucose, ◀ Neuraminic acid.
localization of galactose or LacNAc terminations was impeded by the very low concentrations of high molecular weight glycans.

Different features of the glycosylation pathway could explain the step wise decrease in concentration of the bi-, tri-, tetra- antennary and poly-LacNAc structures. First, the downstream GlcNAc transferases use as substrates the reaction products generated by the transferases situated upstream, which limits the available substrate at each transformation step. Second, the GlcNAc quantity present in the Golgi at any given time could be a limiting factor for synthesis of high molecular weight glycans. The absolute time spent by the nascent glycoproteins in the Golgi may determine the N-glycan remodeling especially because the affinity of GlcNAc transferases for UDP-GlcNAc decreases along the glycosylation pathway.

The profile of methylated N-glycans from Mgat 5+/+ cells supplemented with GlcNAc exhibited the predominant series of high mannose glycans, but also increased concentrations of complex structures (Figure 10). The bi-antennary glycan pool (m/z 2261.3, 2448.3, 2622.3, 2809.3, 2983.4) had a relatively high concentration and was composed mostly of structures substituted at the non-reducing end with neuraminic acid. The oligosaccharides carrying galactose extensions to the [Galβ1-4GlcNAc] unit have very low concentrations or are absent. The tri-antennary sugars have relatively higher concentrations than the ones from Mgat 5+/+ cells grown in low glucose media. The tetra-antennary and poly-LacNAc structures are present in low relative abundances and most of them lack the galactose extensions.
Figure 10. Positive ion MALDI-MS profile of methylated N-glycans from Mgat 5+/+ cells grown in low glucose medium supplemented with GlcNAc • High mannose sugars; ● Mannose, ● GlcNAc, ● Galactose, ▲ Fucose, ◡ Neuraminic acid
A comparison between the high molecular weight glycan profiles from Mgat 5+/+ cells grown in low glucose medium and Mgat 5+/+ cells supplemented with GlcNAc shows the presence of tetra-antennary and poly-N-acetyl-Lactosaminylated structures in both treatments. In Figure 11 the oligosaccharides carrying the galactosyl extension to the [Galβ1-4GlcNAc] unit are marked with the symbol †. The structures marked with 2X have two galactosyl extensions.

Figure 11. Tetra-antennary and poly-N-acetyl-lactosamine structures. 
In the Figure 11, the unmarked peaks correspond to the tetra-antennary and poly-N-acetyl-lactosylated glycans with various neuraminic acid extensions. The reduced frequency of galactose extensions in Mgat 5^{+/+} cells when supplemented with GlcNAc was observed not only for tetra-antennary and poly-LacNAc structures, but also for bi- and tri-antennary ones. This suggests that the increased GlcNAc quantities transported to the Golgi make the GlcNAc transferases more effective in competing with other glycosyltransferases for the acceptor substrate. Although the signal transduction is functional in the cells from both treatments, the receptor glycoprotein – galectin lattice appears to be generated by slightly different structures.

Profiles of methylated N-glycans from Mgat 5^{−−} cells grown in low glucose medium (Figure 12) show the high mannose glycans series in elevated abundances. Also, the truncated sugars synthesized by GnTI and GnTII (m/z 1433.4, 1607.8, 1678.6, 1852.7) were found with increased relative abundances, which suggests that loss of feedback from Mgat 5 modified glycans and defective interactions established in the absence of these structures stimulated the cells to activate the hexosamine pathway as a compensatory mechanism. The bi- and tri-antennary glycan pools were present in relatively low concentrations, but similar to the ones found in Mgat 5^{+/+} cells. The tetra-antennary oligosaccharides, synthesized by the GnTV enzyme, are characterized by molecular weights over 4000 amu and were absent from Mgat 5^{−−} cells (expanded panel). Also, the poly-N-acetyl-lactosaminylated sugars could not be detected in this sample,
Figure 12. Positive ion MALDI-MS profile of methylated N-glycans from Mgat 5^- cells grown in low glucose medium
- High mannose sugars; • Mannose, □ GlcNAc, ● Galactose, ▶ Fucose, ❖Neuraminic acid.
consistent with the LacNAc extensions being attached preferentially to the tetra-
antennary structures synthesized by Mgat 5. Although it is possible for Mgat 5-/-
cells to synthesize bi- and tri-antennary poly-N-acetyl-lactosaminylated sugars,
their very low concentrations, if existent, coupled with the degradative, strong
base conditions imposed by the permethylation reaction could make these
structures fall below the detection limits. Absence of tetra-antennary and poly-
LacNAc oligosaccharides from these cells impairs lattice formation, signal
transduction and the oncogenic potency of PyMT background.

In the profiles of methylated N-glycans from Mgat 5_/- cells supplemented
with GlcNAc, the abundance of high mannose glycan series decreases to
approximately one third of that encountered in the other treatments (Figure 13).
At the same time the relative abundance of complex type glycans increases
dramatically. In these cells, as in Mgat 5^- cells grown in low glucose media, the
products of GnTI (m/z 1433.9, 1608.0) and GnTII (m/z 1679.0, 1853.1) were
highly increased. In addition, stimulation of the hexosamine pathway by the
GlcNAc supplements supplies the cells with large quantities of UDP-GlcNAc and
sustains increased reaction rates for the GlcNAc transferases situated down
stream in N-glycosylation, as well. This generates high concentrations of glycans
with three GlcNAc substituents (m/z 1924.4, 2096.6) and four GlcNAc
substituents (m/z 2343.7). Galactosyl-transferases and neuraminyl-transferases
use these structures as substrates and synthesize olygosaccharides with various
proportions of the neuraminylated [Galβ1-4GlcNAc] unit.
Figure 13. Positive ion MALDI-MS profile of methylated N-glycans from Mgat 5 cells grown in low glucose medium supplemented with GlcNAc. • High mannose sugars; • Mannose, • GlcNAc, • Galactose, • Fucose, • Neuraminic acid.
GlcNAc supplements rescued receptor interaction with the lattice and the cytokine signaling in these cells, but the anticipated galectin-3 preferred ligands were not present in the spectra. Absence of bi- and tri-antennary poly-N-acetyl-lactosaminylated oligosaccharides (expanded panel in Figure 13) suggests that the invasive phenotype manifested by these cells was reinstated by a cell surface lattice generated through alternative structures.

Presence of the glycan bearing four GlcNAc residues attached to the core (m/z 2343) was intriguing. Although it is explainable by the high UDP-GlcNAc concentration which increases the effective reaction rate of GlcNAc transferases, this glycan could not be a tetra-antennary truncated structure because GnTV is not functional. To acquire structural details about this carbohydrate and to confirm peaks annotation the methylated glycan mixtures were submitted to molecular disassembly by collision induced dissociation.
CHAPTER 5

DETAILED STRUCTURAL ANALYSIS BY COLLISION INDUCED DISSOCIATION

Multi-dimensional Mass Spectrometry (MS$^n$) and Collision Induced Dissociation (CID) have been coupled in an ion trap instrument for the isolation and characterization of gas phase components of a complex mixture. Repeated cycles of ion isolation and dissociation in an ion trap can be performed until the large bio-molecules are reduced to their basic units. The bio-molecules can then be identified by interpreting the fragmentation patterns for each MS$^n$ step or by matching them against a fragment library of standard compounds. The resulting spectra reveal molecular composition and structural details regarding a biopolymer sequence as well as branching and linkage position for oligosaccharides (Reinhold et al. 1995; Weiskopf et al. 1997). More recently, features of anomericity and monomer identification have been available using such strategies (Zhang, Singh and Reinhold 2005). These methods have been employed for characterization of linear molecules like DNA (Walters et al. 2002), proteins and peptides (Hunt et al. 1986), as well as more complex structures like glycosphingolipids (Reinhold et al. 1994; Levery 2005) and oligosaccharides (Sheeley and Reinhold 1998; Harvey et al. 1996).
5.1 Multi-dimensional Mass Spectrometric Analysis of the Chicken

Ovalbumin Glycans

Glycans released and profiled by mass spectrometry generate an array of ions representative of each glycoprotein. As an example, the glycoprotein from chicken egg white ovalbumin has only one glycosylation site, but due to microheterogeneity, the released oligosaccharide spectrum exhibits more than 20 different peaks with known isobaric structures (Harvey et al. 2000). For component identification of this complex mixture, multi-dimensional mass spectrometry could be used directly for structural analysis of each individual peak without requiring a previous chromatographic separation.

In MS², the ion of interest is selected from the mass profile and retained in the ion trap, while all other ions are swept away. This precursor ion is then collided with an inert gas and the product fragments are mass profiled. Usually at this level, the fragment ions generated are simple glycosidic bond cleavages. In a series of subsequent MSⁿ steps, product ions are selected and fragmented further with generation of monosaccharide cross-ring cleavages which are informative of linkage and branching patterns. Parallel and successive cycles of isolation and dissociation can generate a tree of structural information with ramifications for each of the parent ion fragments (Zhang et al. 2005).

Multi-dimensional mass spectrometry of the chicken ovalbumin glycan at m/z 2168.8 (Figure 14) uncovers a mixture of four isomers (isobars) within the same ion peak (Geiser et al. 2006). Detailed analysis was pursued in four
Figure 14. Mining glycan structural details by multi-dimensional mass spectrometry (Geiser et al. 2006)
(isolation and CID) steps. During MS² four GlcNAc residues were cleaved (neutral losses) from the non-reducing end as well as one from the reducing-end GlcNAc. The latter residue was distinguished by its different mass which was acquired upon NaBH₄ reduction.

The total mass loss of 1,331 amu in the MS² step generates a product ion, m/z 838, which corresponds to the tri-mannosyl-GlcNAc core. This ion was selected for further dissociation in the MS³ step and generated a series of cross-ring key fragment ions indicative of the branching pattern.

Identification of two pairs of counterpart ions, m/z 662 and 620, as well as m/z 648 and 634 indicates the presence of two pairs of bisected isobars (Figure 15). In MS⁴ of m/z 2168, following the pathway, m/z 2168→838→611, only the tri-mannosyl core was fragmented and the cross ring cleavages of the middle mannose uncovered a set of four isobars with different GlcNAc distributions on the α-1,3 and α-1,6 core mannoses. The ion at m/z 444 indicates presence of a free hydroxyl group on the middle mannose produced by the loss of a bisecting GlcNAc. This study shows that in-depth structural analysis is required for comprehensive carbohydrate identification.

Detailed structural characterization is important because oligosaccharides establish unique interactions with different types of lectins and modulate various biological processes. Many lectins recognize specific glycan structures or conformations, types of monosaccharides or linkage positions. Acquiring detailed structural information about the lectin's ligands is of great value in understanding the types of interactions they generate and the biological functions they regulate.
Figure 15. Cross ring cleavages generated in MS$^4$: m/z 2168 – 838 – 611
5.2 Structural Analysis of Bi-, Tri- and Tetra-antennary Glycans by Collision Induced Dissociation

The complex glycan mixture released from total cell proteins in the Mgat 5 treatments makes it difficult to unambiguously identify the oligosaccharide's structures. Collision Induced Dissociation was employed for identifying the N-linked glycans and for resolving the carbohydrate peaks with identical molecular weight values and composition but with different structures referred to as isobars. Also, CID was used to support peak identification predicted by GlycoMod and to resolve between the multiple hits to GlycoSuite Data Base.

Collision Induced Dissociation in the MALDI QIT instrument provides adequate sensitivity for low sample quantities. However, a comprehensive analysis using repeated fragmentation cycles during multi-dimensional mass spectrometry requires elevated concentrations of oligosaccharides because the interfering background noise increases with the decrease of glycan concentration.

The decreasing activity of GlcNAc transferases as the glycosylation pathway advances produces much lower quantities of the high molecular weight compounds. Correlated with the requirements for tandem mass spectrometry, structural determination of low abundance high molecular weight glycans becomes a challenging problem. For this, the spectra were acquired either manually by conducting the laser shots in areas rich in desired analyte or automatically by accumulating a few thousands profiles for one spectrum.
In Mgat 5 cells the partially and fully decorated oligosaccharides generate very rich methylated spectra. Although the tetra-antennary and poly-N-acetyl-lactosaminylated structures could be identified in the Mgat 5\(^{+/+}\) cells, these were absent from Mgat 5\(^{-/-}\) cells. An initial hypothesis suggested that in an Mgat 5 deficient background, the GlcNAc supplements would reestablish the lattice interaction and signal transduction by facilitating biosynthesis of bi- and tri-antennary poly-N-acetyl-lactosaminylated glycans. These types of structures could be identified neither in Mgat 5\(^{+/+}\) cells nor in Mgat 5\(^{-/-}\) cells. However, due to similarities in their monosaccharides composition the bi-antennary glycans with one N-acetyl-lactosamine extension are molecular weight isomers with the tri-antennary glycans. In the same way, the tri-antennary glycans with one N-acetyl-lactosamine extension are isomers with tetra-antennary ones. Also, the bi-antennary sugars with two N-acetyl-lactosamine extensions are isomers with the tetra-antennary glycans. To resolve these isomeric structures and the multiple alternatives introduced by GlycoMod predictions and to identify the role of bi- and tri-antennary poly-N-acetyl-lactosaminylated sugars in establishing the cell surface lattice, various glycans from the different Mgat 5 treatments were subjected to CID.

Within a mass tolerance of only two Daltons a GlycoMod search gave two different hits in GlycoSuite DB for the molecular ion at m/z 2983 (Figure 12). One possible composition corresponds to a bi-antennary mature glycan \((\text{Hex})_2 (\text{HexNAc})_2 (\text{Deoxyhexose})_1 (\text{NeuAc})_2 + (\text{Man})_3 (\text{GlcNAc})_2\) with two neuraminylated branches attached to the N-glycan core \((\text{Man})_3 (\text{GlcNAc})_2\) and a
fucosylated reducing end. The second possibility is a tetra-antennary without any neuraminic acid or fucose substituents \((\text{Hex})_4 (\text{HexNAc})_4 + (\text{Man})_3(\text{GlcNAc})_2\) (Figure 16).

\[
\text{(Hex)}_2 (\text{HexNAc})_2 (\text{Deoxyhexose})_1 (\text{NeuAc})_2 + (\text{Man})_3(\text{GlcNAc})_2 \quad \text{and} \quad (\text{Hex})_4 (\text{HexNAc})_4 + (\text{Man})_3(\text{GlcNAc})_2
\]

*Figure 16.* N-glycans predicted by GlycoMod for m/z 2983.

To identify the structure of the N-glycan at m/z 2983 the parent ion was fragmented by CID. The molecular dissociation profile shows successive neutral losses of two neuraminic acid residues and two \([\text{Gal-GlcNAc}]\) units (Figure 17). The fragmentation pattern generates a specific core ion at m/z 867 and identifies this N-glycan as a bi-antennary structure. It has been shown before that during molecular disassembly experiments the predominant ion peaks are generated by cleavage of the most labile glycosidic bonds established by GlcNAc and neuraminic acid residues (Zaia 2004).
Another GlycoMod search proposed two structures for the ion m/z 3023.

One possible structure (Hex)9 + (Man)3(GlcNAc)2 could be the initial high mannose glycan GlcNAc\textsubscript{2}Man\textsubscript{3}Glc\textsubscript{3} transferred by the oligosaccharyltransferase complex from the dolichol-phosphate to the nascent proteins in the ER lumen (Figure 2). A second proposed structure could be either a bi-antennary or a tri-antennary glycan (Hex)1(HexNAc)3(Deoxyhexose)1(NeuAc)2 + (Man)3(GlcNAc)2.

MS\textsuperscript{2} dissociation of the ion m/z 3023 shows specific fragment ions and neutral losses indicative of a bi-antennary structure (Figure 18). Although, the GalNAc – GlcNAc group (Lac-di-NAc) is not very common in mammals, the fragmentation pattern proves its presence in a bi-antennary glycan.
Methylated oligosaccharides exhibit a preferential rupture proximal to the reducing end of HexNAc that generates a mixture of B-type and Y-type ions (Domon and Costello 1988) with approximately equal abundances (Egge et al. 1983). The glycosidic rupture induces formation of an unsaturated bond on the non-reducing terminal ion and a free hydroxyl group on the reducing terminal ion. An exception from this pattern is cleavage of the 1-3 linkage which ruptures on the reducing side of the glycosidic oxygen leaving the point of unsaturation on the reducing terminal ion. These marks left behind after glycosidic bond breakage give information about branching and linkage. Monitoring these ions and neutral loss fragments greatly aids in understanding of structure. For example, a terminal monosaccharide neutral loss acquires a unique mass when...
positioned at the reducing end, due to unsaturation. Internal residues acquire two modulations in mass, due to glycosidic ruptures at the reducing end and the non-reducing end. Also, each ramification point imposes an additional alteration in the neutral loss mass.

Molecular weight calculation and empirical observations of fragmentation patterns from many other bi-antennary structures (results not presented) show that glycans with two substituted hydroxyl groups on the core generate a specific tri-mannosyl ion at m/z 866 (Figure 17 and Figure 18). This ion served as a structural fingerprint for all glycans with two GlcNAc substitutions on the core because it was produced independent of antenna extension or reducing end fucosylation.

When colliding the ion peak at m/z 3618, three neutral losses of terminal neuraminic acid residues were observed (Figure 19). Also, the molecule lost three internal Gal-GlcNAc units and a reducing-end GlcNAc. The trimannosyl core fragment ion m/z 853 indicated three free hydroxyl groups at the non-reducing end which is indicative of the number of antennae and one double bond at the reducing end GlcNAc. This fragmentation pattern and the specific core ion identified the structure at m/z 3618 to be a tri-antennary not fucosylated oligosaccharide.
Figure 19. Tri-antennary glycan – MS² m/z 3618; • Mannose, ● GlcNAc, • Galactose, ▼ Fucose, ○ Neuraminic acid.

Unlike the previous structure, fragmentation of the oligosaccharide at m/z 3823 showed a neutral loss of a fucosylated reducing end GlcNAc (Figure 20). Also, the molecule lost two terminal N-acetyl-neuraminic acid residues, one terminal N-glycolyl-neuraminic acid and three intercalated Gal-GlcNAc residues. This summation provides strong indication for a tri-antennary structure. Although one of the antennae carries a different substitution than the previous structure and the reducing end is fucosylated, the tri-mannosyl core ion has the same molecular weight. This shows that, independent of its composition, a glycan bearing three substituents on the core, including the tri-antennary sugars, can be reduced to the same fingerprint ion (m/z 853).
Figure 20. Tri-antennary glycan – MS² m/z 3823; • Mannose, • GlcNAc, • Galactose, ▲ Fucose, ◇ Neuraminic acid.

N-Glycolyl neuraminic acid is an analog of N-acetyl neuraminic acid and has one extra hydroxyl group which gains a methyl group after methylation (Figure 21). The two analogues could both be referred to as sialic acids.

Figure 21. Different neuraminic acid residues.
N-glycolyl neuraminic acid is a common component of glycoconjugates in murines and other mammals and participates in formation of the blood group epitopes in canines and felines. Also, N-glycolyl neuraminic acid is recognized and modulates binding of different viruses in animals (Vihinen-Ranta et al. 2004). The hydroxylase that modifies this monosaccharide is expressed in great apes, but the enzyme and its product are absent from human cells generating variations in susceptibility to viral infections among primates (Muchmore et al. 1998). Also, traces of N-glycolyl neuraminic acid were found in tumor cells in humans and the monosaccharide has been investigated as a possible cancer biomarker (Varki 2002).

For oligosaccharides of large size, comprehensive branching and linkage determination requires three or four multi-dimensional mass spectrometry steps and relatively large sample concentrations. Most of the high molecular weight glycans were present in the Mgat 5 cells in very low concentrations (see relative abundances of glycans at m/z 3023, 3823 and the tetra-antennary sugars in Figures 9, 10, 12 and 13) and do not allow numerous steps of molecular fragmentation. Although not a detailed analysis, the structural fingerprinting method described above resolves between bi-antennary glycans with one N-acetyl-lactosamine extension and tri-antennary isomers, as well as tri-antennary glycans with one N-acetyl-lactosamine extension and their tetra-antennary isomers.

Characteristic fingerprint ions were identified in the tetra-antennary glycan pool. For example, dissociation of the glycan at m/z 4445 indicated four
alternative fragmentation patterns, three of which are marked in Figure 22 and one is left unmarked.

Figure 22. Tetra-antennary glycan – MS² m/z 4445; • Mannose, • GlcNAc, • Galactose, > Fucose, ○ Neuraminic acid.

Antennae heterogeneity and alternative cleavage sites introduced by the Gal-Gal unit generate a rich fragment ion density in this spectrum. Neutral losses during dissociation identify four terminal residues and three internal Gal-GlcNAc disaccharides. Independent of the cleavage succession, all fragmentation pathways converge to the fingerprint ion at m/z 1288.3. The tri-mannosyl core has three free hydroxyl groups at the non-reducing end and one Gal-GlcNAc unit with one free hydroxyl, which means that the glycan has four substituents.
attached to the core. Together with the fragmentation patterns, the fingerprint ion identifies the oligosaccharide as a tetra-antennary structure.

Similar dissociation patterns were observed for the glycan at m/z 4603 (Figure 23), although the spectrum shows fewer fragment ions due to identical mass of each antennae, which create a homogeneous non-reducing side. During molecular disassembly the molecule loses the fucosylated reducing end, four N-acetyl-neuraminic acid residues and three Gal-GlcNAc units. The remaining tri-mannosyl core was identical to the one found in previously described structure (Figure 22) and was considered a fingerprint ion for the tetra-antennary sugars.

Figure 23. Tetra-antennary glycan – MS² m/z 4603; •Mannose, •GlcNAc, •Galactose, ▲Fucose, ◆Neuraminic acid.
The low concentration of the high molecular weight glycans does not permit a multiple step molecular dissociation. The ion fingerprinting method allowed identification of almost all glycans in the Mgat 5 tumor cells (results not presented). Also, possible isomers with the poly-N-acetyl-lactosaminylated structures could be differentiated with ease using this strategy. For example, the glycan at m/z 5052.3 in the Mgat 5√√ GlcNAc supplemented cells has a relative abundance of less than 1% from the maximum abundance. However, the fragmentation pattern shows an identical core ion as the structure at m/z 4603 and also an extra neutral loss of a Gal-GlcNAc unit. These findings identify the glycan at m/z 5052 as a tetra-antennary glycan with one N-acetyl-lactosamine extension and not a penta-antennary isomer (Figure 24).

![Figure 24. Isomer identification for m/z 5052.](image)

Screening the glycan mixtures from the Mgat 5 tumor cells with different genetic backgrounds and GlcNAc treatments identified bi-, tri- and tetra-antennary glycans as well as poly-N-acetyl-lactosaminylated tetra-antennary structures in both GlcNAc treatments for Mgat 5√√ cells. A similar analysis for the Mgat 5√√ cells showed only bi- and tri-antennary glycans and none of the tetra-
antennary structures synthesized by GnTV or the tetra-antennary poly-N-acetyl-lactosaminylated ones.

An initial hypothesis suggested that the GlcNAc supplements to the Mgat 5\sup+\sup tumor cells could rescue the lattice interaction by facilitating addition of poly-N-acetyl-lactosamine extensions to the bi- and tri-antennary glycans. Although cytokine signal transduction was restored by the GlcNAc supplements, none of the poly-LacNAc bi- or tri-antennary glycans could be identified in the Mgat 5\sup+\sup GlcNAc supplemented cells.

The poly-N-acetyl-lactosaminylated oligosaccharides show low concentrations in the Mgat 5\sup+/\sup cells and perform their biological activities though interactions of high affinity rather than high avidity. The branch GlcNAc β-1,6 Man α-1,6 Man initiated by Mgat 5 activity is not required for addition of poly-N-acetyl-lactosaminylated extensions. However, this branch generates preferred substrates for GnT(i), the GlcNAc transferase which adds the poly-LacNAc extensions and promotes carcinogen transformation (Pierce and Arango 1986; Dennis et al. 1987). This metabolic bias generates an increase in poly-N-acetyl-lactosaminylation for the Mgat 5 modified glycans in comparison to the bi- or tri-antennary sugars. Also, the lower affinity of GnT(i) for bi- and tri-antennary glycans explains the absence of poly-LacNAc extensions on these structure in the Mgat 5\sup+\sup tumor cells. Although the detection of these structures was not possible their presence in the Mgat 5\sup+\sup cells cannot be totally excluded. However, if bi-antennary and tri-antennary glycans with poly-LacNAc extensions are synthesized in these cells, they are present in extremely low concentrations and
may not be able to reestablish lattice formation and signal transduction by themselves (Lau. et al. in prep.)

These findings forced us to reevaluate the mechanisms of lattice formation and to search for alternative structures, which could rescue signal transduction in Mgat 5<sup>-/-</sup> tumor cells grown in GlcNAc supplemented medium.

### 5.3 GlcNAc Transferase Products Compete or Cooperate?

The molecular dissociation experiments allowed partial structural assignments for most of the oligosaccharides found in Mgat 5 tumor cells and an identification of high molecular weight galectin-3 ligands, which establish the cell surface lattice in Mgat 5<sup>+/+</sup> cells (Partridge et al. 2004). However, the structures that rescued signal transduction in Mgat 5<sup>-/-</sup> cells supplemented with GlcNAc could not be identified.

A reevaluation of the native and methylated glycan spectra pointed out the major differences among the four Mgat 5 tumor cell treatments. The tetra-antennary and poly-N-acetyl-lactosaminylated structures were found in both GlcNac treatments for Mgat 5<sup>+/+</sup> cells, but not in the lattice deficient Mgat 5<sup>-/-</sup> cells grown in low glucose media. This demonstrates their essential role in modulating signal transduction by establishing the galectin – receptor glycoprotein superstructure. At the same time, the poly-N-acetyl-lactosaminylated glycans were absent from the Mgat 5<sup>-/-</sup> cells rescued by GlcNAc supplements. This shows that poly-N-acetyl-lactosaminylation is not favored in an impaired Mgat 5
background. These findings suggest that the GlcNAc supplements to Mgat 5⁻⁻⁻ cells reestablished the lattice formation by promoting biosynthesis of alternative structures with similar properties.

A search for large variations in oligosaccharide properties among the four Mgat 5 GlcNAc treatments showed an important difference in the amount and distribution of the glycans carrying the Gal-Gal-GlcNAc antenna between the Mgat 5⁺⁺⁺ cells grown in low glucose media and the ones supplemented with GlcNAc. The terminal galactose residue was found preponderantly in Mgat 5 cells grown in low glucose media, while the GlcNAc supplemented cells had a higher incidence of neuraminic acid terminal residues. It is known that neuraminic acid capping can influence various biological phenomena, including cell-cell interactions, binding of toxins, viruses, bacteria, and parasites to their cellular receptors, as well as maintaining stability and biologic activity of hormones or enzymes in vivo (Oetke et al. 2002). It may be possible that the terminal neuraminic acid is favored for lattice binding and could offer an advantage in establishing signal transduction in GlcNAc supplemented samples. However, the cell surface superstructure was functional in both Mgat 5⁺⁺⁺ GlcNAc treatments, although the glycans display marked differences in terminal capping. This makes it difficult to estimate the role of the different termini in the interactions with galectin-3, but suggests a lattice's plasticity and its ability to interact with alternative structures.

One major variation among the four types of analyzed cells and GlcNAc treatments was the extensive increase in the relative abundance of glycans with
one, two, three and four GlcNAc residues attached to the trimannosyl core in Mgat 5^−/− GlcNAc supplemented cells. It would be expected to see these N-glycans in elevated concentrations because these are the direct products of GlcNAc transferases and the GlcNAc supplements sustain their biosynthesis by generating large quantities of high-energy donor molecules. However, the glycan with four GlcNAc substituents, a possible precursor to a tetra-antennary structure, was not expected in these cells due to the Mgat 5 null background. A tandem mass spectrometry analysis of the sugars with three and four GlcNAc residues attached to the core (m/z 2098 and 2343, in methylated spectra) from the Mgat 5^−/− GlcNAc supplemented cells identified their structure and raised additional questions.

Collision induced dissociation of the glycan at m/z 2098 showed in MS² successive neutral losses of a fucosylated reducing end and three non-reducing GlcNAc termini (Figure 25). Also, this fragmentation generates the fingerprint ion for glycans with three core substituents (m/z 852.5). A predicted molecular structure and the fragmentation points are depicted in Figure 26. The fragment at m/z 1838 could be produced by glycosidic cleavage of either of the terminal GlcNAc residues. Although the MS² fragmentation informs about the monosaccharide composition, it could not identify the distribution of the GlcNAc substituents on the core mannose residues. To accomplish this, the core ion was dissociated in the MS³ step.
**Figure 25.** MS² at m/z 2098.

**Figure 26.** Possible structure for the glycan at m/z 2098.

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Dissociation of the core ion in MS\(^3\) showed fragmentation patterns indicative of a mixture of two isomers with different substituent distribution for the core mannoses (Figure 27). The ion pair at m/z 662 and 648 indicated that the \(\alpha\)-1,3 and \(\alpha\)-1,6 core mannoses establish glycosidic bonds with a different number of substituents and suggest the presence of a tri-antennary structure. At the same time the ion pair at m/z 458 and 444 indicated that two isomers are present, one has the position \(\beta\)-1,4 of the middle mannose involved in a glycosidic linkage and a second isomer has a methylated hydroxyl group at the same position. The fragmentation patterns of the core ions are presented in Figure 28 and 29.

Figure 27. Core ion fragmentation; MS\(^3\) m/z 2098 – 853.
Figure 28. Core ion fragmentation for the tri-antennary precursor.

Figure 29. Core ion fragmentation for the bisected glycan.
One component of the mixture was the predicted tri-antennary precursor with two GlcNAc residues attached to the $\alpha$-1,3 core mannose and the third GlcNAc residue linked to the $\alpha$-1,6 core mannose (Figure 28). A second component present in the mixture carries one GlcNAc residue attached to the $\alpha$-1,3 core mannose, the second GlcNAc residue linked to the $\alpha$-1,6 core mannose and the third GlcNAc linked in $\beta$-1,4 of the core middle mannose (Figure 29). This bisecting GlcNAc is attached by GlcNAc transferase III (GnTIII) activity (Narasimhan 1982) and its presence on the core inhibits the activity of other glycosyl transferases as well as mannosidases, rendering the glycan of an unprocessed type (Schachter 1986).

When colliding the ion at m/z 2343 the fragmentation pattern showed neutral loses of a fucosylated reducing end and also loss of four terminal GlcNAc residues (Figure 30). The proposed structure for this bisected glycan was depicted in Figure 31.

The core ion at m/z 838 was dissociated in MS$^3$ (Figure 32) and indicated losses of core mannoses with one substituent (m/z 634) and two substituents (m/z 648). Presence of another key fragment ion (m/z 444) showed that the core middle mannose has three substituents attached. These are the $\alpha$-1,3 and $\alpha$-1,6 mannoses and the $\beta$-1,4 bisecting GlcNAc (Figure 33). Absence of the ion at m/z 458 (Figure 28) from the MS$^3$ fragmentation showed that the peak at m/z 2343 was generated by a single oligosaccharide with a bisecting GlcNAc core.
Figure 30. $\text{MS}^2 \text{ m/z 2343}$

Figure 31. Proposed structure for the bisected glycan at m/z 2343.
Figure 32. Core ion fragmentation MS\(^3\) m/z 2343 – 838.

Figure 33. Core ion fragmentation for the bisected glycan at m/z 2343
Identification of a single bisected glycan at m/z 2343 showed that, in the absence of GnTV activity, the Mgat 5\(^{-/-}\) cells cannot synthesize the tetra-antennary structures and that the GlcNAc supplements cannot overcome the energy activation barrier for this enzyme. However, GlcNAc supplementation increases the activity rate of all other GlcNAc transferases including the bisecting enzyme GnT III.

Expression of GnT III enzyme was found to be elevated in brain and kidney cells from mice and humans, but was present at relatively low levels in other tissues (Bhattacharyya et al. 2002). Regulation of GnT III activity and its glycan products proved to be tissue specific. In Mgat 5\(^{-/-}\) cells grown in low glucose medium, the bisected glycans were present at very low concentrations, which shows that these structures are not required for signal transduction. The GlcNAc supplemented cells, and especially the Mgat 5\(^{-/-}\) ones, showed increased levels of bisected glycans, which brings into question their role in signal transduction.

The increased relative abundance of bisected glycans in Mgat 5\(^{-/-}\) GlcNAc supplemented cells could be only a byproduct of the elevated concentrations of UDP-GlcNAc, which stimulate all GlcNAc transferase activity. At the same time, GnT V and GnTIII compete in the trans Golgi for the same acceptor substrate, the bi- and tri-antennary precursors (Figure 3). GlcNAc supplements, together with the GnT V deficiency in Mgat 5\(^{-/-}\) cells, can produce a phenocopy of GnT III over-expression with various implications in cell functions. For example, over-expression of GnT III suppressed lung metastasis of melanoma cells by inhibiting
GnTV activity (Yoshimura et al. 1995). Also, expression of both GnT III and GnT V genes is enhanced during hepatocarcinogenesis, although they are not expressed in the normal liver (Taniguchi et al. 1999). In the same time, GnT III transfectants produce spleen carcinoma by offering protection against natural killer cell cytotoxicity (Yoshimura 1996). Taken together these findings suggest that the bisected glycans can sustain or inhibit cancer progression through different cell type specific mechanisms. Although GnT III and GnT V enzymes compete for the same substrate, the interactions established by their glycan products can converge or antagonize in supporting or inhibiting specific biological functions.

The role of bisected glycans in lattice formation and signal transduction, if any, is poorly understood in Mgat 5 cells. Other experimental approaches may be required for assessing the effects of enzyme competition and the interactions established by their products.
CHAPTER 6

IDENTIFICATION OF ALTERNATIVE GALECTIN-3 LIGANDS
IN Mgat 5+ GlcNAc SUPPLEMENTED CELLS

Mass spectrometric analysis of methylated oligosaccharides from different GlcNAc treatments of Mgat 5 tumor cells allowed identification of GnT V products and the galectin-3 ligands in Mgat 5+/+ cells. At the same time, an absence of poly-N-acetyl-lactosaminylated glycans from the Mgat 5−/− cells implies that lattice formation and signal transduction were modulated through alternative interactions in the GlcNAc supplemented samples.

Evaluation of the quantitative and qualitative changes in the oligosaccharide distribution from Mgat 5−/− GlcNAc supplemented cells showed a substantial decrease in high mannose glycans concomitant with an increase in complex type glycans. This shift in the high mannose/complex glycan ratio could be explained by an increase in glycan remodeling through stimulation of Golgi GlcNAc transferases and the hexosamine pathway caused by the GlcNAc supplements. Another explanation could be that the GlcNAc supplements sustain activity of Golgi GlcNAc transferases including the activity of GlcNAc-1-phosphotransferases. This may increase the rate of biosynthesis and transfer of lipid-linked oligosaccharides to proteins and elevate the number of occupied glycosylation sites for a given glycoprotein. In the last possibility, the GlcNAc
supplements may influence the total number of glycans attached to proteins as well as the number of complex type sugars. However, lattice formation and signal transduction were influenced by the number of occupied glycosylation sites, as well as the type of glycan structures present on the receptor glycoproteins, in other words the density of galectin ligands.

The bioinformatics study by Lau et al. (in prep), which compares the number of glycosylation sites on the extracellular domain of cell surface receptors, showed that the receptors involved in cell differentiation and morphogenesis have fewer glycosylation consensus sites than the growth and proliferation signal receptors. This implies that the activity of the two categories of receptors will be affected differently by the interaction with the lattice. For example, the transforming growth factor-β (TGF-β) receptors TβRI and TβRII have only one and three N-X-S/T consensus sites, respectively. Mgat5−/− cells displayed a two- to threefold decrease in sensitivity to TGF-β compared with approximately 100-fold decrease in sensitivity to EGF, PDGF and IGF (Partridge et al. 2004). At the same time, Mgat5−/− cells supplemented with GlcNAc displayed a full rescue of receptor sensitivity to TGF-β and only a 70% rescue of receptor sensitivity to anabolic factors (Lau et al. in prep). These results suggest that the large number of glycosylation sites on the anabolic receptors modulates the receptor response to cytokines and the lattice sensitivity to GlcNAc supplements in a gradual manner. Also, the limited number of glycans on morphogenic receptors causes an ultrasensitive lattice dependency on GlcNAc supplements and a switch-like receptor activation mechanism.
To identify the N-linked glycans that generate the lattice interactions and to determine their quantitative variations, Mgat 5+/+ and Mgat 5−/− cells were grown in media supplemented with increasing concentrations of GlcNAc. The series of GlcNAc titrations were identical for both Mgat 5 genetic backgrounds, with increasing GlcNAc concentrations from 0 to 50 mM.

The oligosaccharides were released from the individual cell cultures, reduced and directly MS profiled. The native glycan profiles are a more accurate quantitative representation for neutral oligosaccharides as this avoids ulterior derivatization and purification which could introduce degradative reactions or contaminants. However, the oligosaccharides released from the Mgat 5 cells were a mixture of neutral and acidic species with different ionization properties. In positive ion extraction, neutral glycans ionize as Na+ molecular ions [M+Na]+, while the acidic glycans ionize as deprotonated ions [M-H]− and go undetected. This makes it difficult to detect both ion types in a quantitative manner. Glycan methylation resolves the ionization problem through esterification of the carboxyl groups, but could introduce variables associated with derivatization and sample handling. Also, the methylated glycan profiles show that most of the high molecular weight sugars are present in very low concentrations, which makes it difficult to obtain a quantitative estimation.

For these reasons the oligosaccharides from the Mgat 5 cells were desialylated by non-specific mild acidic hydrolysis. Neuraminic acid release provides a comparable product for all components in the mixture and consolidates under one ion peak similarly branched glycans. The signal strength...
of glycans ionized by MALDI appears to reflect accurately the amount of material on the target, providing that the correct matrix was chosen (Harvey 1993). The matrix used for ionization of oligosaccharides in Mgat 5 tumor cells was 2,5-dihydroxy benzoic acid (DHB), which is known to produce a linear response over several orders of magnitude (Harvey 1999). A quantitative evaluation of a standard oligosaccharide dilution series mixed with another standard oligosaccharide with a constant concentration shows a direct dependence between the peak intensity and the compound concentration as well as the intensity variation between the two compared ion peaks (results not shown).

The glycans from each Mgat 5 genetic background were extracted and profiled before and after neuraminic acid release. The comparison between the two sets of spectra provides information about the ratio between the low molecular weight non-sialylated and the high molecular weight sialylated glycans. A quantitative evaluation was performed among all of the eight GlcNAc treatments for each titration series.

The glycans released from Mgat 5+/+ cells and their molecular structures are represented in Figure 34. The oligosaccharide profiles before and after neuraminic acid release for three of the GlcNAc treatments from the Mgat 5+/+ titration series are presented in Figures 35, 36, and 37.
Figure 34. Native N-glycans from M2at 5−/−, after neuraminic acid release.

M2at 5−/− 0 mM GlcNAc
Figure 35. Native N-glycans from Mgst 5+/+ grown in low glucose; A – before neuraminic acid release; B – after neuraminic acid release.
**Figure 36.** Native N-glycans from Mga5^{+/+} with 31 mM GlcNAc supplement; A – before neuraminic acid release; B – after neuraminic acid release.
Figure 37. Native N-glycans from Mga5^+/+ with 50 mM GlcNAc supplement; A - before neuraminic acid release; B - after neuraminic acid release.
In all of the spectra the high mannose glycans are marked with a dot for an easier comparison. The profiles of native oligosaccharides before neuraminic acid release are presented in the upper part of the Figures 35, 36 and 37. All of these profiles show elevated concentrations of high mannose type sugars and relatively low concentrations of complex type glycans. The oligosaccharide profiles after neuraminic acid release are presented in the lower part of Figures 35, 36 and 37. Although the concentrations of high mannose glycans remain relatively constant, the release of neuraminic acid determines accumulation of bi-, tri- and tetra-antennary glycans under elevated common ion peaks. Tetra-antennary and poly-N-acetyl-lactosaminylated structures were identified in these spectra but in smaller number after neuraminic acid release. The greatest variation in relative abundance was observed with the cumulated bi-antennary glycans (m/z 1811). The glycan profiles for the other GlcNAc treatments from the titration series are presented in the Appendix B.

The structures of native N-glycans from Mgbt 5−/− cells grown in low glucose media are depicted in Figure 38. The native glycans profiles for the same three GlcNAc treatments as those presented for the Mgbt 5+/+ cells are shown in Figures 39, 40 and 41. The glycan spectra before and after neuraminic acid release are shown in the upper and lower part of the figures, respectively. In all spectra the high mannose glycans are marked with a dot.
Figure 38. Native N-glycans from Mgat 5<sup>−/−</sup>, after neuraminic acid release.
Figure 39 Native N-glycans from Mgat 5\(^{-}\) grown in low glucose; A – before neuraminic acid release; B – after neuraminic acid release.
Figure 40. Native N-glycans from Mga t 5' with 31 mM GlcNAc supplements; A – before neuraminic acid release; B – after neuraminic acid release.
Figure 41. Native N-glycans from Mgat $5'$ with 50 mM GlcNAc supplements; A – before neuraminic acid release; B – after neuraminic acid release.
The glycan profiles of Mgat 5^{-} cells grown in low glucose media showed a predominant high mannose glycan series similar to Mgat 5^{+/+} cells. However, a comparison among the eight GlcNAc treatments (profiles of other GlcNAc treatments are presented in the Appendix B) showed an increasing relative abundance for the complex glycans in parallel with a decreasing concentration for the high mannose glycans.

The ratio between complex and high mannose glycans increased in the titration series and the complex glycans became the predominant peaks in the profile of Mgat 5^{-} cells supplemented with 31 mM GlcNAc (Figure 40). The relative abundance of N-glycans from the Mgat 5^{+/+} and Mgat 5^{-} cells' titration series were expressed as percents of the total added values and tabulated in Appendix C. The percent values of different classes of N-linked glycans (bi-antennary, tri-antennary and high mannose type) were averaged and their ratio variation is presented in Figures 42 and 43.

![Figure 42. Relative abundance variation for bi-antennary N-glycans](image-url)
The ratio between the averaged relative abundances of bi-antennary and high mannose glycans is represented in Figure 42 as a function of the GlcNAc concentration in the titration series. The bi-antennary/high mannose glycan ratio showed relatively small variations for the Mgat 5+/+ titration series and a slight increase in the cells supplemented with elevated GlcNAc concentrations. In Mgat 5−/− cells the same ratio showed a continuous increase as the GlcNAc supplements increase. The ratio doubles its value in the cells supplemented with 37.5 mM GlcNAc and then registers a drop for the higher GlcNAc concentrations (Figure 42).

The relative abundance values for tri-antennary glycans were averaged and expressed in relation to the averaged high mannose values. This ratio is plotted in Figure 43 as a function of the GlcNAc concentrations.

![Figure 43. Relative abundance variation for tri-antennary N-glycans](image)

The tri-antennary/high mannose glycan ratio shows little variation in the Mgat 5+/+ cells. However, the same ratio exhibits a step-wise increase in Mgat 5−/− titration
series with maximum values for the highest GlcNAc supplement. The elevated
tri-antennary glycan proportions in Mgat 5'_/_ cells supplemented with 43.75 and
50 mM GlcNAc could explain the decrease of the bi-antennary/high mannose
ratio for the same GlcNAc concentrations (Figure 42). An elevated Golgi
remodeling could generate an increased density of tri-antennary structures to the
detriment of their precursors — the bi-antennary glycans and would suggest a
constant number of occupied glycosylation sites.

The products of GlcNAc transferases increase in concentration along the
Mgat 5'_/_ cells titration series (m/z 1488, 1690, 1894). This generates elevated
concentrations of bi-antennary and tri-antennary glycans.

The tetra-antennary glycans were absent from Mgat 5'_/_ cells, and very low
levels of poly-N-acetyl-lactosaminylated structures were detected. This suggests
the tetra-antennary glycans are preferred for the addition of N-acetyl-lactosamine
extensions. However, the poly-LacNAc structures were either present in trace
amounts or absent from these cells, suggesting that other oligosaccharides may
have rescued the lattice interaction.

A comparison among all eight GlcNAc treatments shows that the relative
abundance of bi-antennary and tri-antennary glycans increases in the cells
supplemented with higher concentration of GlcNAc (m/z 1649, 1853, 2014,
2030). This implies that the surface of Mgat 5'_/_ cells supplemented with GlcNAc
presents a higher density of bi- and tri-antennary glycans. Although some of
these structures are not mature glycans, they carried at least one Gal-GlcNAc
unit recognized by galectin-3. These analogs are not preferred ligands for
galectin-3, like poly-LacNAcs, but they appear to interact with this lectin and participate in lattice formation. I propose that the elevated concentrations of bi- and tri-antennary sugars may overcome the deficit of Mgat 5 modified glycans and their poly-LacNAc derivatives and establish cell surface interactions with adequate avidity to maintain cytokine receptor activation and signal transduction.

To illustrate the variations in concentrations for different glycan types their relative abundances were summed and expressed as fractions of the total oligosaccharide pool. The fractions for each glycan category were plotted as a function of GlcNAc supplement concentration from the Mgat 5\(^{-}\) titration series (Figure 44). The graphic shows a decrease in high mannose glycan levels in parallel with an increase in tri-antennary structures. Although the concentrations of bi-antennary glycans exhibited variation among different GlcNAc treatments, overall they were maintained at a steady level. Also a slight increase in concentration of bisected oligosaccharides could be observed. The spectra and the graph showed that the most extensive variation in N-linked glycan processing imposed by the GlcNac supplements was the distortion of the high mannose / complex glycan ratio and the substantial increase in tri-antennary structures.

The mass spectrometric analysis was used to confirm a computational simulation of N-glycan processing performed by our collaborators (Figure 45). The mathematical model takes into consideration the Golgi N-glycan processing, the distribution of receptor glycoforms and the lattice dependent regulation of surface receptor.
Figure 44. Quantitative mass spectrometry analysis of Mgat 5^{+} glycans.

Figure 45. Mathematical prediction of glycan distribution in Mgat 5^{+} cells.

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The model predicts an increase of tri-antennary structures attached to cytokine receptors with an increase in GlcNAc concentrations, which correlates with the experimental mass spectrometry results.

This comparative study shows that cytokine signaling can be modulated through the interactions established by the cell surface receptor. The receptor association with the lattice is dependent on genetic influences such as Mgat 5 expression and N-glycan processing by GnT V, which produces high affinity ligands for galectin-3. Also, cell surface superstructure formation can be influenced by environmental factors such as high concentrations of available GlcNAc. This stimulates the hexosamine pathway, which sustains synthesis of complex type glycans with lower content of N-acetyl-lactosamine (bi- and tri-antennary glycans) but in higher concentrations. Together, these factors participate in balancing a regulatory mechanism, which controls discriminatively activation of morphogenic receptors with ultrasensitive kinetics and of anabolic receptors in a sensitive, graded manner.
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APPENDICES
Table 1A. Proposed structures for methylated N-glycans

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<tr>
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| 43 | 4603.3 | ((\text{Hex})_5 (\text{HexNAc})_4 (\text{Dhex})_1, (\text{NeuAc})_3 + (\text{Man})_3 (\text{GlcNAc})_2) |
| 44 | 4691.3 | ((\text{Hex})_5 (\text{HexNAc})_5 (\text{Deoxyhexose})_1 (\text{NeuAc})_3 + (\text{Man})_3 (\text{GlcNAc})_2) |
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APPENDIX B

GlcNAc Titration Series

The spectra presented in this section represent native N-linked glycans from Mgst 5 cell lines grown in low glucose media supplemented with increasing GlcNAc concentration. The GlcNAc concentrations in the titration series were: 0 mM, 12.5 mM, 18.75 mM, 25 mM, 31.25 mM, 37.5 mM, 43.75 mM and 50 mM.

All figures in this section show profiles of native N-glycans before neuraminic acid release in the upper part and after neuraminic acid release in the lower part.
Figure 46B. Native N-glycans from Mga5^{+/+} grown in low glucose
Figure 47B. Native N-glycans from Mgat 5** with 12.5 mM GlcNAc supplement
Figure 48B. Native N-glycans from Mgat 5+/+ with 18.75 mM GlcNAc supplement
Figure 49B. Native N-glycans from Mgtat 5^{-/-} with 25 mM GlcNAc supplement
Figure 50B. Native N-glycans from Mga5+/+ with 31.25 mM GlcNAc supplement
Figure 51B. Native N-glycans from Mgat 5+/+ with 37.5 mM GlcNAc supplement
Figure 52B. Native N-glycans from Mga t 5+/+ with 43.75 mM GlcNAc supplement
Figure 53B. Native N-glycans from Mgtat 5+/+ with 50 mM GlcNAc supplement
Figure 54B Native N-glycans from Mgst 5−/− grown in low glucose
Figure 55B. Native N-glycans from Mgat 5+, with 12.5 mM GlcNAc supplements.
Figure 56B. Native N-glycans from Mga5<sup>−/−</sup> with 18.75 mM GlcNAc supplements
Figure 57B. Native N-glycans from Mgtat 5−/− with 25 mM GlcNAc supplements
Figure 58B. Native N-glycans from Mga5°/ with 31 mM GlcNAc supplements
Figure 59B. Native N-glycans from Mgt 5', with 37.5 mM GlcNAc supplements
Figure 60B. Native N-glycans from Mga 5+ with 43.75 mM GlcNAc supplements
Figure 61B. Native N-glycans from Mgat 5−/− with 50 mM GlcNAc supplements
APPENDIX C

Relative Abundance Variation of N-glycans in the GlcNAc Titration Series

The relative abundances of N-linked glycans profiled along the GlcNAc titration series are tabulated in this section. This values were used for quantitative estimations.
Table 2C. Relative abundance variation of N-linked glycans with the increased GlcNAc concentration

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