β1,6GlcNAc-BRANCHED N-GLYCANS REGULATE INFLAMMATION and CANCER

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Graduate Department of Medical and Molecular Genetics, University of Toronto

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ABSTRACT

Glycosylation of cell-surface and secreted glycoproteins is the most complex type of post-translational modification, consuming a large portion of cellular resources, and may have as broad an impact on metazoan biology as phosphorylation. β1,6GlcNAc-branched asparagine (N)-linked oligosaccharides, the biosynthesis of which requires the activity of β1,6N-Acetylgalcosaminyltransferase V (GlcNAc-TV), constitute a subset of structurally heterogeneous glycans found on cell surface and secreted glycoproteins. Increased β1,6GlcNAc-branching of N-glycans has been linked to cancer progression and metastatic potential. In addition, the expression of GlcNAc-TV is induced by activation of Src and Ras.

We first examined the expression pattern of Mgat5, the gene encoding GlcNAc-TV, during murine development. GlcNAc-TV transcripts were absent in embryonic tissues at E7, but became expressed throughout the embryo at E9.5, and then progressively restricted to regions of the developing central nervous system and to the epithelia of skin, intestine, kidney, endocrine tissues and respiratory tract. A common feature of cells in basal epithelia and in the cortical neural epithelium is the capacity to migrate, a cellular function, which may require GlcNAc-TV-dependent glycoconjugates.

To examine the function of GlcNAc-TV dependent glycosylation in murine biology, we generated mice deficient in this enzyme by targeted mutation of the Mgat5 locus. Homozygous Mgat5−/− mice were viable, fertile. These mice lacked β1,6GlcNAc-branched N-glycans, indicating that only one gene encodes GlcNAc-TV activity. GlcNAc-TV deficient mice displayed enhanced hypersensitivity to skin irritants. However, the onset of the inflammatory response was slower, likely due to a reduced rate of leukocyte migration in vivo, which was associated with increased adhesion to substratum, measured in vitro. GlcNAc-TV deficient T cells, but not B cells, were hypersensitive to mitogenic stimulation. Our results suggest that β1,6GlcNAc-branched N-glycans on the T cell surface have a direct role as a negative regulator of T cell response to antigen, thus contributing to shutting down immune responses.

In order to study the role of GlcNAc-TV in metastatic breast cancer, we crossed GlcNAc-TV deficient mice with mice transgenic for the polyoma middle T antigen (PyMT) under the
regulation of a mammary specific promoter. PyMT is a potent oncogene that interacts and activates Shc, Src and PI3 kinase. Signaling through these pathways increases Mgat5 expression, making this a relevant model for studying the function of GlcNAc-TV in cancer. GlcNAc-TV deficiency caused a delay in the development of palpable mammary tumors, the area of which grew at a rate 6.7-fold slower than did tumors of wild type littermates, resulting in 4.5-fold reduction in tumor weight. GlcNAc-TV deficiency also resulted in >95% reduction in the incidence of lung metastases. Our results suggest that GlcNAc-TV activity may enhance the formation and turnover of focal adhesions, thereby increasing signaling via the integrin/PI3 kinase/PKB pathway, and influencing cell migration and metastatic potential.
Acknowledgements

I would like to thank my supervisor, Jim Dennis, who has given me a great project to work on. While giving many helpful suggestions, Jim also allowed me enough freedom to explore different avenues and to make mistakes of my own devising.

I am grateful to my committee, Drs. Andras Nagy and Harry Schachter for their attention and comments. They were always available for discussion, and provided a different slant on some of the problems that were facing me during this project.

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Finally, although I promised my parents to leave them out of the acknowledgements, this list would be glaringly incomplete without them. So that's it. Just thank you.
Attribution of Data

I am responsible for all the data that is presented in this thesis with the following exceptions. The lectin histochemistry shown in Figure 2 of Chapter 2 was done by Dr. Robert Campbell. Immunofluorescence images of fibroblasts and tumor cells shown in Figure 6 of Chapter 3 and Figure 8 of Chapter 4 and the Northern blot shown in Figure 1B of Chapter 4 were generated by Judy Pawling. Western blots, shown in Figure 9 of Chapter 3, and in Figure 9 of Chapter 4 were done by Dr. James Dennis. Jamie Fata stained mammary pads for histology, cell proliferation and apoptosis (Figure 2B, 5A-D and table 1 of Chapter 4).
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- Cell Migration and metastasis
- Sialylation and Metastasis
- Endogenous Lectins and Tumor Cell Adhesion
- Host anti-tumor immunity
- Carbohydrate processing inhibitors as anti-cancer agents

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GlcNAc-TV

THESIS OUTLINE

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<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>α-MEM</td>
<td>alpha-minimal essential medium</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>CD</td>
<td>cluster designation</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed-type hypersensitivity</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence aided cell sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>Gal</td>
<td>galactose</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GlcNAc-TV</td>
<td>β1,6N-Acetylglucosaminytransferase V</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation sequence motif</td>
</tr>
<tr>
<td>LAT</td>
<td>Leukocyte</td>
</tr>
<tr>
<td>LFA-1</td>
<td>lymphocyte function-associated antigen</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>PH</td>
<td>plekstrin homology</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SA</td>
<td>sialic acid</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor-CD3 complex</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>X-gal</td>
<td>4-chloro-5-bromo-3-indolyl-β-galactoside</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>zeta-chain associated protein</td>
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CHAPTER 1: General Introduction
INTRODUCTION

Glycosylation of cell-surface and secreted glycoproteins is the most complex post-translational modification. As such, it requires a large portion of cellular resources. For example, an estimated 0.5 to 1% of the vertebrate translated genome is devoted to glycosylation-related genes (Varki and Marth, 1995), a commitment similar in magnitude to that of protein phosphorylation. Analysis of somatic glycosylation mutants and transgenic mice suggests that the functional impact of glycosylation on metazoan biology might be as broad as that of phosphorylation as well. Glycosylation modulates and regulates a variety of protein-protein interactions relevant to cellular proliferation, adhesion and migration. The structural complexity of glycans has impeded functional analysis of specific structural subgroups, but targeted mutations in mice affecting specific steps in the biosynthetic pathways of glycosylation offer a powerful tool for studying such structure-function relationships. This thesis describes the results of ablating a specific subset of N-glycan structures by targeting Mga5 the gene encoding β1,6N-Acetylglucosaminyltransferase V (GlcNAc-TV), which catalyzes the addition of the β1,6GlcNAc-branch on N-glycans.

Oligosaccharides on glycoproteins are classified by the nature of their linkage to the protein as either N-linked or O-linked (reviewed in (Kornfeld and Kornfeld, 1985; Schachter, 1986)). N-glycans are the focus of this work (for biosynthesis, see Figure 1). Dolichol-mediated N-glycosylation is absent in bacteria, but present in all eukaryotic species, including yeast. Glycosylation aids the proper folding of glycoproteins and the mediation of chaperone-protein recognition in the endoplasmic reticulum (ER) of eukaryotes (Velders and Kast, 1999; Ware et al., 1995). A glucosyltransferase recognizes
misfolded glycoproteins and reglucosylates the polypeptide so it is recirculated for degradation (Velders and Kast, 1999).

The activity of GlcNAc-TI, which is essential for the synthesis of hybrid- and complex-type N-glycans (Schachter, 1986), is absent in yeast but present in all multicellular organisms studied to date. Thus, GlcNAc-TI demarcates a fundamental evolutionary boundary between uni- and multicellular organisms. This divergence suggests a role for complex and hybrid N-glycans in intercellular communication and in the interactions of the cell with its environment. Consistently, complex- and hybrid-type glycosylation occurs in the Golgi network, an organelle devoted to secretory and cell-surface proteins.

N-glycosylation can be viewed as consisting of four phases (Figure 1). Transfer of a pre-assembled precursor oligosaccharide from a dolichol pyrophosphate to the nascent polypeptide in the rough ER is followed by trimming by glycosidases in the rough ER and cis-Golgi. The trimmed intermediate passes into the medial-Golgi, where branching by GlcNAc-Ts is added to the growing structure that is then elongated and capped in the trans-Golgi. The structural variability of glycans is dictated by tissue-specific regulation of glycosyltransferase genes, acceptor and sugar nucleotide availability in the Golgi, compartmentalization, and by competition between enzymes for acceptor intermediates during glycan elongation. Several genes encoding glycosyltransferases appear to be regulated by multiple promoters, and glycosyltransferase activity levels measured in vitro do not necessarily reflect message levels, suggesting post-transcriptional and/or post-translational regulatory mechanisms (Lo and Lau, 1996; Perng et al., 1994; Saito et al., 1995; Yang et al., 1994). Given the possible combinations of available monosaccharides, linkages, branching and variable
lengths of glycan chains, the potential for structural diversity of glycans in metazoan cells is very large.

Glycosyltransferases catalyze the transfer of a monosaccharide from specific sugar nucleotide donors onto a particular hydroxyl position of a monosaccharide in a growing glycan chain in one of two possible anomeric linkages (either α or β). The majority of glycosyltransferases characterized to date show exquisite specificity for the donor and acceptor they recognize and the linkages they catalyze. The protein microenvironment in which the immature glycan chain is synthesized also affects glycosyltransferase catalytic efficiency (Do et al., 1994; Granovsky et al., 1994) and leads to structural heterogeneity of glycans between glycoproteins, and even between different glycosylation sites on individual glycoproteins produced by the same cell (Rademacher et al., 1988; Sheares, 1988). A range of biosynthetically related glycan structures may be present at any particular glycosylation site of a mature glycoprotein, creating a population of "glycoforms". The prevalence of a particular glycoform can affect the intrinsic biological activity of an individual glycoprotein. For example, the peptide hormones lutropin and erythropoietin are produced with structurally diverse glycans and the discrete glycoforms of these hormones have significantly different serum half-life and potency in vivo (Fiete et al., 1991; Takeuchi and Kobata, 1991). Cytokine and adhesion receptors are similarly subject to glycoform heterogeneity with the potential to modify the kinetics of physical association of these receptors, and thereby intracellular signaling.

The Golgi biosynthetic pathways appear to have evolved to generate structural diversity on glycoproteins, but the selective pressure favoring such resource-expensive complexity of glycosylation remains unclear. One possible explanation is parasite
**Figure 1:** Schematic of N-linked glycan biosynthesis showing the Golgi compartments.

A high-mannose pre-assembled precursor oligosaccharide is transferred from a dolichol pyrophosphate to the nascent polypeptide in the rough ER. It is trimmed by glucosidases and by α-mannosidase I in the rough ER and cis-Golgi. The action of GlcNAc-TI is required for the production of hybrid- and complex-type N-glycans. The action of α-mannosidase II or α-mannosidase III, followed by GlcNAc-TII is required to synthesize the precursor to all complex-type N-glycans. Note that GlcNAc-TIII substitutes the core β-mannose residue and redirects the pathway into “bisected N-glycans”, blocking subsequent action of GlcNAc-branching transferases (Schachter, 1986). The terminal sequences are added to both N- and O-linked glycans by β1,4Gal-T, β1,3GlcNAc-T(i), β1,6GlcNAc-T(i), α2,3ST, α2,6ST and α1,3Fuc-T. GlcNAc-TV is one of several medial-Golgi enzymes that initiate a specific branch (the β1,6GlcNAc branch) that is variably elongated in the trans-Golgi generating structural diversity in the mature N-glycans. The GlcNAc-TV product is the preferred intermediate for extension with polylactosamine chains (i.e. Galβ1,4GlcNAcβ1,3 repeating units of 2 to >10 in length). Polylactosamine synthesis is also regulated by β1,3GlcNAc-T(i) activity, glycoprotein transit time in the trans-Golgi, and competition by chain-terminating enzymes including α1,2Fuc-T and α2,6SA-T. The polylactosamine and Lewis antigens that preferentially elongate the β1,6GlcNAc-branch are expanded in the grey box.

Abbreviations used are: oligosaccharyltransferase, OT; the α-glucosidases, GI, GII; the β-N-acetylglucosaminyltransferases, TI, TII, TIII, TIV, TV, T(i) and T(I); the α1,2mannosidases, MI, α1,3/6mannosidases MII, MIII; β1,4-galactosyltransferases (Gal-T), α-fucosyltransferases (Fuc-T), α-sialyltransferases (ST).
Figure 2: Schematic of O-linked glycan biosynthesis.

O-glycan specific enzymes are shown in the shaded area. Unlike N-glycosylation, O-glycosylation is initiated directly on the nascent polypeptide, without an intermediate. The terminal sequences shown in the non-shaded area are added to both N- and O-linked glycans by a common set of enzyme as noted in Figure 1. The numbers in front of the sialyltransferases (STs) indicate the linkage of the transferred SA. Note that core 2 GlcNAc-T initiates an antenna required for addition of polylactosamine and polyvalent Lewis antigens. GalNAc in mucins can also be substituted with β1,3GlcNAc and in several other linkages creating “other cores” disaccharides, which mature differently. Abbreviations are polypeptide O-α-N-acetylgalactosaminytransferase, (ppGT); β1,3galactosyltransferase, (3Gal-T); the core 2, β-N-acetylglycosaminytransferases, (C2-T).
Other terminal sequences

polylactosamine

T(l) Fuc-T T(l)

SLeX

"l"-branched

H-antigen

GlcNAC □  GalNAC □  Gal ●  Fuc ▽  SA ♦
evasion. Glycan binding to lectin is a common means of adhesion between multicellular organisms and parasitic organisms. For example, adhesion of *Helicobacter pylori* to the mucous and gastric epithelial cells is mediated by fucosylated blood-group antigens of the O blood-group, and has been associated with a greater incidence of ulcerative disease in O blood group individuals (Boren et al., 1994). Infection by *Entamoeba histolytica*, which can result in colitis and dysentery, is mediated by recognition of Gal and GalNAc residues by a parasite-expressed lectin (Dodson et al., 1999), and *Tritrichomonas foetus*, an infectious bovine parasite associated with inflammation, abortion and infertility, adheres to its host by a sialic-acid specific lectin (Babal and Russell, 1999). However, different terminal sequences on glycans can also mask lectin-binding sites for parasite infection. Thus, continuous generation of molecular diversity in metazoans may enhance population fitness in the ongoing battle to evade parasitic organisms.

Newly arising glycan structures are co-opted, on occasion, into functions unrelated to pathogen evasion. The conserved structures become the acceptors for newly evolved glycosyltransferases, and this generates additional diversity. In this scenario, the older the capacity for a particular carbohydrate structure, the more likely it is that the structure has acquired biological functions intrinsic to the organism, and unrelated to pathogen evasion. Consistently, mutations in ancient parts of the glycosylation pathway will affect many downstream oligosaccharide structures, some of which now perform developmental functions. Indeed, the phenotypes of glycosyltransferase-deficient mice are progressively milder for enzymes operating later in the biosynthetic pathway. GlcNAc-TI deficient (*Mgat1<sup>-/-</sup>*) mice lack all hybrid- and complex-type N-glycans, and they die between embryonic day (E) 8.5 and E9.5 due to multiple organ failure. Mutations in downstream genes, affecting only subsets of complex-type N-glycans are less severe. *Mgat2<sup>-/-</sup>* mice survive until birth, and *Mgat3<sup>-/-</sup>, Mgat5<sup>-/-</sup>*.
GalT1<sup>−/−</sup> and FucTVII<sup>−/−</sup> mice are viable (Asano et al., 1997; Chui et al., 1997; Granovsky et al., 1997; Ioffe and Stanley, 1994; Maly et al., 1996; Metzler et al., 1994; Priatel et al., 1997).

The effects of glycosylation on cellular proliferation and migration have been studied mostly in the context of tumor progression and metastasis. Previously, over 40 somatic mutants of N-glycosylation enzymes have been isolated and characterized (Stanley, 1983). Glycosylation mutant tumor cells grow autonomously in culture, but in vivo, solid tumor growth and/or experimental metastasis are compromised by certain mutations. Cancer growth and metastasis involve many cell-cell interactions including cell migration, invasion, differentiation, angiogenesis and immune system evasion. As such, one or more of these processes would appear to require precise glycosylation of macromolecules for efficient tumor growth and metastasis.

**GLYCOSYLATION AND CANCER**

Malignant transformation is often associated with altered glycosylation of glycoproteins and glycolipids, and expression of certain glycan structures in tumors correlates with clinical prognosis (reviewed in (Hakomori, 1996; Kobata, 1998)). Glycans are structural components of many cell surface glycoprotein receptors, such as cell adhesion receptors and cytokine receptors, the signaling of which controls fundamental cellular processes that are of prime interest when studying cancer progression and metastasis. The role of carbohydrates in cancer initiation, progression and metastasis is an area of intense study using somatic tumor cell mutants, tumor cells transfected with glycosyltransferase genes and transgenic mice.
**Cancer initiation and progression:** The study of cancer genetics is key to our understanding of the disease, and provides a context in which to consider glycosylation effects. Multiple genetic changes, combined with selection pressure in the host environment, provide conditions for tumor formation and progression. Mutations that impart growth advantages are maintained and selected for in the cellular population, allowing mutant cells to preferentially expand during tumor progression and acquisition of metastatic potential (Cairns, 1981). For example, the pre-neoplastic cells of aberrant crypt foci in colon display increased proliferation: a cancer-predisposing condition because these rapidly cycling cells, with increased DNA replication, are more likely to sustain subsequent rare mutations. These rare mutations allow aberrant crypt foci cells to form pre-malignant polyps, which can progress to adenomas and finally to metastatic colon carcinomas (Lengauer et al., 1997).

Cancer mutations result in either loss-of-function in a gene product, designated “tumor suppressor proteins” (e.g. p53, APC, WT1), or missense mutations which activate a “proto-oncogene” (e.g. h-ras). Tumor suppressor genes have also been described as “caretaker” and “gatekeeper” genes, controlling genomic integrity and aspects of cellular proliferation, respectively (reviewed in (Kinzler and Vogelstein, 1997)). The retinoblastoma gene, Rb-1, is a gatekeeper gene, responsible for maintaining cells in G1 phase (Weinberg, 1999). Loss-of-function mutations in Rb-1 promote entry into the S phase of the cell cycle, and subsequent tumorigenesis in retinal epithelial cells. The mismatch DNA repair enzymes MSH2 and MLH1 are caretaker genes and their inactivation in colon cancers leads to mutations in other genes that can enhance growth, such as the TGF-β receptor type II gene (Grady et al., 1999). P53 and other genes that ensure faithful segregation of chromosomes during mitosis also serve a caretaker function (Cross et al., 1995). Heritable mutations in tumor suppressor genes such as P53, Rb-1,
and APC greatly increase the risk of cancer, as only one wild-type allele is present and therefore only one somatic inactivating mutation is required for complete loss of function. Some of the heritable mutations common in human cancers were engineered in mice to facilitate the study of the pathways leading to oncogenesis (reviewed in (Jacks, 1996; Webster and Muller, 1994)). Indeed, many of these transgenic mice show increased incidence of pre-malignant lesions and tumors, and thereby are useful in establishing cause and effect relationships in the cascade of events leading to metastatic cancer.

Currently, genes encoding glycosylation-related enzymes are not included in the catalogue of somatic and heritable mutations responsible for cancer initiation or progression. However, changes in glycosylation are commonly observed in human carcinomas, and in cell lines transformed with activated Ras and v-Src (Easton et al., 1991; Lu and Chaney, 1993; Pierce and Arango, 1986; Yamashita et al., 1985; Yamashita et al., 1985; Yamashita et al., 1985). In earlier studies of tumor cell glycosylation mutants, α2,3sialylation, increased polylactosamine content and β1,6GlcNAc-branching of N-glycans were suggested to enhance tumor growth and metastasis in mice (Dennis, 1986; Dennis et al., 1987; Finne et al., 1982; Lu et al., 1994). More recently, over-expression of glycosyltransferase genes in tumor cell lines, and studies in glycosyltransferase deficient mice confirm that cancer-associated changes in glycosylation play a causal role in cancer progression and metastasis.

**Tumor Cell Proliferation:** The ras proto-oncogene sustains activating mutations in approximately 20% of all human tumors (Velders and Kast, 1999). In addition, Ras signaling is induced by other common mutations such as amplification of Neu/ErbB-2 in breast cancer (Denhardt, 1996). Signaling by the Ras GTPase activates Raf-1 kinase, which leads to the activation of AP1 (i.e. c-Fos/c-Jun dimers) and Ets transcription
factors (Wasylyk et al., 1998). These transcription factors regulate expression of multiple genes involved in cell cycle progression and cell motility, as well as metalloproteases, growth factors and glycosyltransferases.

The relationship between Ras and c-Fos was demonstrated genetically. Application of skin carcinogens results in invasive skin tumors in mice transgenic for activated Ras. However, c-Fos deficient mice carrying the activated v-H-ras transgene develop benign tumors only, in which matrix metalloproteases (MMPs) and vascular endothelial growth factor (VEGF) transcripts are suppressed (Saez et al., 1995). VEGF induces host endothelial cells to develop microvasculature, thereby providing the necessary oxygen and nutrients to the expanding tumor. MMPs secreted by tumor cells digest extracellular matrix and facilitate tumor cell invasion through extracellular matrix that separates tissue compartments. Thus, according to this experiment, Ras acts upstream of c-fos, which is an effector of VEGF and MMP transcription. This study is an example of how genetic analysis facilitates the ordering of specific molecules in a signaling cascade.

The Ras signaling pathway is activated by growth factors including members of the EGF, PDGF and NGF families (Chao, 1992). In Mgat3 transfected U373 glioma cells, EGF receptor levels and signaling were observed to decrease, and both the EGF receptor and the NGF receptor, Trk, were substrates of GlcNAc-TIII-dependent glycosylation (Rebbaa et al., 1997). Dimerization and phosphorylation of Trk were also reduced in Mgat3 transfected PC12 cells (Ihara et al., 1997). Conversely, overexpression of the O-glycan β1,6GlcNAc-bran ching enzyme, core 2 GlcNAc-T in PC12 cells enhanced Trk receptor O-glycosylation, signaling via MAPK, and PC12 differentiation (Personal communication, C.E.Warren). Trk receptor levels on the cell surface, as well as receptor activity are enhanced by β1,6GlcNAc-bran ching of either N- or O- glycans.
Therefore, it is likely that a common feature, such as polylactosamine extensions may be involved in stabilizing the receptor in some manner.

NGF/Trk and EGF/EGFR both activate Ras/MAPK in PC12 cells but with different cellular outcomes. NGF/Trk activation results in differentiation, whereas EGF/EGFR activation results in proliferation (Tan and Kim, 1999). The apparent dichotomy can be resolved by considering the kinetics of activation through these receptors, which are different. The studies with MgaT3 and core 2 GlcNAc-T transfected cells suggest that glycosylation may modify intracellular signaling and that GlcNAc-TIII-mediated glycosylation and β1,6GlcNAc-branching of either N- or O-glycans exert opposite effects on Trk signaling. GlcNAc-TIII and GlcNAc-TV, which catalyzes the addition of the β1,6GlcNAc-branch on N-glycans, compete for glycan acceptor intermediates and the action of GlcNAc-TIII renders glycan intermediates poor substrate for GlcNAc-TV in *in vitro* biochemical studies (Schachter, 1986). Indeed, B16 melanoma cells transfected with a GlcNAc-TIII expression vector showed an increase in hybrid-type glycans and a reduction in GlcNAc-TV products (Sheng et al., 1997). Thus, the relative levels of GlcNAc-TIII and GlcNAc-TV in a particular cell may provide kinetic fine-tuning to the all-or-none phenomenon of Trk dimerization and signaling.

In many cancer cells, where Ras is constitutively activated, the observed effects of glycosylation are probably due to their modulation of signaling through receptors utilizing other pathways. Activating mutations in ras, of the type found in human tumors, bypass the need for growth factors and cell surface glycoprotein receptors. Therefore, the glycosylation status of glycoprotein receptors and cytokines upstream of Ras may be less significant in cells with a mutated ras gene, as the cells acquire a large measure of growth-factor independence. A similar phenomenon was recently demonstrated for the cytokine pathway initiated by Wnt / Frz signaling in *Drosophila*. 
Wnt receptor (Frz) signaling requires UDP-glucose dehydrogenase, which produces glycosaminoglycans, required as co-receptors. Inactivating mutations in β-catenin and APC, which are negative regulators of the growth stimulation by the Wnt pathway, circumvent the requirement for Wnt / Frz, and for UDP-glucose dehydrogenase (Cumberledge and Reichsman, 1997).

**Cell Migration and metastasis:** Metastatic carcinoma cells are not restricted by the tight junctions characteristic of normal epithelial cells. With the transition from normal to malignant status, cell-cell adhesion molecules are switched off, and substratum adhesion is modified to optimize focal adhesion turnover and cell motility. In normal epithelial cells, tight junctions and desmosomes associate with cytoskeleton intermediate filaments to maintain cell shape and polarity. Critical to the maintenance of these contacts is Ca²⁺-dependent homophilic binding of the cell-adhesion molecule E-cadherin in the zones of adhesion (Christofori and Semb, 1999). Germline mutations in the E-cadherin gene have been found in cases of familial gastric cancer, and either E-cadherin expression or its function is suppressed in sporadic carcinomas of most tissues (Birchmeier, 1995; Fri xen et al., 1991). Forced over-expression of E-cadherin in tumor cells suppresses tumor growth in mice (Birchmeier, 1995). E-cadherin and CD44 were subject to GlcNAc-TIII-dependent N-glycosylation in transfected B16 cells (Sheng et al., 1997; Yoshimura et al., 1996), and levels of E-cadherin increased in GlcNAc-TIII transfectants. Therefore, inactivating mutations, reduced gene expression or aberrant glycosylation of the gene product can cause suppression of E-cadherin activity, and the subsequent enhancement of tumor cell growth autonomy. The over-expression of the hyaluronic acid-binding cell adhesion receptor CD44 suppresses tumor progression (Schmits et al., 1997). CD44 activity is glycosylation-dependent, as enzymatic removal of sialic acid and galactose from complex-type N-glycans of CD44 enhances binding to hyaluronate (Skelton et al., 1998). GlcNAc-TIII transfection
of B16 melanoma cells enhanced CD44-mediated cell adhesion, reduced invasion through extracellular matrix in vitro, and reduced lung colonization in vivo when transfected cells were injected intravenously into mice (Sheng et al., 1997; Yoshimura et al., 1995). These studies demonstrate that GlcNAc-III-mediated glycosylation may enhance tumor cell adhesion, thereby reducing tumor cell growth autonomy. This outcome may be achieved partly by competing for substrate with GlcNAc-TV and reducing β1,6-branching of N-glycans, which have been implicated in reducing adhesion and promoting cell migration.

Metastatic tumor cells must migrate over extracellular matrix (ECM) to make their escape from the primary tumor, then gain access to the blood stream, attach to the distal vascular bed, migrate over ECM and grow in the secondary organ (Nicolson, 1988). Tumors of various origins differ greatly in phenotypes that affect metastasis. Various steps in the metastatic process, such as cell migration on ECM, required for both escape from the primary tumor and seeding at the secondary site, can be rate limiting for metastasis, depending on the tumor phenotype. Cell migration on ECM is mediated primarily by signaling through the integrin receptors. These receptors mediate attachment to substratum, aggregating into focal adhesions when bound to the ECM glycoproteins fibronectin, laminin and collagen (Ruoslahti, 1996). In growth-arrested non-transformed cells, ligand-engaged integrins form stable adhesion plaques with links to actin stress fibers inside the cell. In either growth factor stimulated cells or transformed cells, focal adhesions induce continuous recruitment of signaling complexes on the cytosolic side of the plasma membrane. This results in turnover of the actin microfilaments, and activation of the Ras/MAPK and PI3K/PKB signaling pathways (Jaln et al., 3653; Schlaepfer et al., 1995). Cell migration rates depend upon optimal turnover of focal adhesion complexes. Integrin receptor levels and activity are balanced against ECM adhesion domains to regulate focal adhesion turnover and thereby migration.
rates. Substratum density changes can vary reciprocally to integrin levels or their affinities (Palecek et al., 1997). As such, rates of focal-adhesion turnover and cell motility exhibit bell-shaped responses to changing ligand or receptor levels. Focal adhesion turnover also stimulates intracellular signaling and cell proliferation via PI3K and c-Src kinases, which creates a positive feedback loop.

Studies in Mv1Lu cells, S115 mammary carcinoma cells and T cells from patients with Sezary syndrome indicate that β1,6GlcNAc-branched N-glycans are present on α5, α, and β1 integrin subunits (Braut-Boucher et al., 1998; Demetriou et al., 1995; Leppa et al., 1995). Cellular adhesion to ECM inversely correlates with β1,6GlcNAc-branching, while the motility of the cells correlates with β1,6GlcNAc-branching directly (Demetriou et al., 1995). Therefore, it is possible that integrin-mediated signaling is subject to regulation by β1,6GlcNAc-branched N-glycans.

*Sialylation and Metastasis:* Terminal N- and O-glycan sequences added in the trans-Golgi compartment contribute to malignancy, as suggested by studies on tumor cell glycosylation mutations. MDAY-D2 mutants, over-expressing α2,6SA-T due to a retroviral insertion into the gene promoter, showed 3-10 fold fewer metastases and 60% slower tumor growth (J. Lau, personal communication). The mutant cells had predominantly α2,6SA rather than the wild-type α2,3SA on the cell surface (Takano et al., 1994). Similarly, transfection of a glioma cell line, U373 MG cells with α2,6SA-T reduced invasion (Yamamoto et al., 1997). The hyposialylated CMP-NeuNAc-hydroxylase-expressing MDAY-D2 mutants gave rise to slower growing solid tumors than did the parent cells. In addition, loss of sialylation in B16 melanoma mutants due to over-expression of α1,3Fuc-T, α1,2-Fuc-T or α1,3Gal-T, resulted in loss of metastatic potential (Dennis, 1986; Finne et al., 1982; Gorelik et al., 1997; Yamamoto et al., 1997). These results imply that capping
N-acetyllactosamine with N-acetylneuraminic acid, specifically in the α2,3 linkage, may be conducive to efficient metastasis.

**Endogenous Lectins and Tumor Cell Adhesion:** The GlcNAc-TV enzyme product in the N-glycan pathway and core 2 GlcNAc-T product of the O-glycan pathway are preferred intermediates for extension with polylactosamine chains, which add heterogeneity in the form of polymer length and various capping sequences (Cummings and Kornfeld, 1984; Schachter, 1986; Yousefi et al., 1991). Galectins binding to N-acetyllactosamine, are widely expressed, and have been implicated in tumor cell adhesion during metastatic spread (Perillo et al., 1998). Galectins 1 and 3 are expressed on the surface of B16 melanoma, the UV-2237 fibrosarcoma and the K-1735 melanoma cells, and have previously been shown to facilitate organ colonization and metastasis by blood-borne tumor cells (Ohannesian et al., 1995). Intravenous infusion of Gal or arabinogalactan inhibited liver colonization by murine tumor cells, presumably by blocking their retention to the microvasculature (Beuth et al., 1987). UDP-Gal transporter mutants lack polylactosamine in both O- and N-glycans, and show the most severe attenuation of tumor growth and metastasis. Restoring Gal to the surface of UDP-Gal transporter mutants, using bovine β1,4Gal-T, increased tumor cell adhesion to non-activated endothelial cells and enhanced metastasis of the mutants in mice (Cornil et al., 1990). Genetic revertants of the UDP-Gal transporter mutation also regain the malignant phenotype (Dennis and Laferte, 1986).

The Lewis carbohydrate antigens Le^a^, Sialyl-Le^a^, Le^y^ and SLe^a^ are often over-expressed in human carcinomas and have been shown to mediate attachment of colon tumor cells to selectins *in vitro* (Mannori et al., 1995). Polylactosamine forms the backbone of the dimeric Le^a^ and Le^y^ antigens, and dimeric Le sequences correlate with poor prognosis in colon cancers (Hoff et al., 1989). E- and P- selectins on endothelial cells bind SLe^a^ and
related sequences found on core 2 GlcNAc-branched O-glycans (Li et al., 1996). Core 2 GlcNAc-T transcripts increase in human colon carcinomas, as do Fuc-TIV and ST3Gal II, both of which are required for the biosynthesis of Sle\(^x\) (Ito et al., 1999; Shimodaira et al., 1997). Indeed, inhibition of core 2 GlcNAc-T activity and GalNAc-Ser/Thr substitution \textit{in vivo}, by treating tumor cells with benzyl-\(\alpha\)-GalNAc reduces organ colonization (Bresalier et al., 1991).

The normal function of selectin ligands, including Sle\(^x\), is the mediation of leukocyte attachment to blood vessel endothelial cells, an important step for leukocyte extravasation (reviewed in (Vestweber and Blanks, 1999)). Leukocytes express selectin ligands on cell surface glycoproteins like PSGL-1, which may not be expressed by cancer cells. However, on cancer cells, selectin ligands may be overexpressed on other glycoproteins, and thus may still be able to contribute to metastasis \textit{in vivo}. Additionally, by expressing selectin ligands, blood-borne tumor cells can aggregate with platelets and leukocytes, which may express L-selectins and can mediate attachment indirectly. Forced expression of E-selectin in the liver of transgenic mice enhanced metastasis of Sle\(^x\)-expressing B16F10 melanoma cell to the liver, rather than to their usual destination of the lung (Biancone et al., 1996). However, it is unclear that attachment of blood-borne tumor cells to endothelium is a rate-limiting step in clinical metastasis for most tumors. Patients with ascites tumors treated with peritoneal shunts to maintain their salt balance push millions of peritoneal tumor cells into the circulation, but this does not significantly increase the number of metastases observed at autopsy (Jamjoom et al., 1993).

\textbf{Host anti-tumor immunity:} Tumor-specific antigens are widely present but whether they elicit meaningful anti-tumor immune response is still open for debate. For example, in normal tissues, The O-glycan Tn and T antigens (GalNAC-polypeptide and Gal\(\beta\)1,3GalNAc-polypeptide, respectively) are shielded by subsequent branching or
capping, but ~90% of these epitopes are uncovered in tumors. A twenty-year study suggests that immunization with purified T/Tn antigen in adjuvant and a trace of the hyperantigen phosphoglycolipid A results in significantly improved 5 and 10-year survival (Springer, 1999). However, the study was conducted on a limited number of patients with tumor grades ranging from II to IV, all of whom were receiving additional treatment, such as chemotherapy or radiation therapy. Thus, the benefit of T/Tn vaccination remains controversial.

Tumors produce cytokines such as TGF-β and IL-10 that act to suppress cell-mediated immunity (i.e. Th1 responses) (Botti et al., 1998), and prevalent carbohydrate epitopes, such as Le^x and polylactosamine may also suppress Th1 responses. Le^x and polylactosamine sequences, found in Schistosoma mansoni eggs, have been shown to suppress Th1 response required to clear the infection (Palanivel et al., 1996). Susceptible mice mount the ineffective humoral Th2 response, which leads to tissue damage and continued infection (Palanivel et al., 1996). Thus, it is suggested that Lewis sequences and polylactosamine on tumor cells may also suppress Th1 cellular immunity and T cell production of INF-γ and IL-2 cytokines that can reduce the growth of some tumors.

**Carbohydrate processing inhibitors as anti-cancer agents:** The alkaloids swainsonine and castanospermine block tumor cell metastasis and invasion through extracellular matrix in vitro. Swainsonine is a competitive inhibitor of Golgi α-mannosidase II, which blocks the N-glycan biosynthetic pathway prior to β1,6GlcNAc-branched, and results in production of hybrid-type glycans (Figure1). Swainsonine-treated cells showed increased transcription rates for tissue inhibitor of metalloproteinases (TIMP-1). Swainsonine also suppresses MMP-2 expression in human tumor cells, a metalloproteinase associated with cancer progression in humans.
Swainsonine has been particularly useful for anti-cancer studies due to its apparent lack of toxicity (reviewed in Goss et al., 1995). Swainsonine has been tested in two phase I clinical trials with encouraging results that show both low toxicity and evidence of clinical responses. Phase II trials designed to measure efficacy of oral swainsonine treatment in renal cell carcinoma are currently being done. Swainsonine should be considered a first generation compound, with room for improvement. Swainsonine inhibits lysosomal α-mannosidases with potency equal to that of the Golgi α-mannosidase II, and therefore induces lysosomal storage, and complex-type N-glycan biosynthesis can proceed via alternate pathways that circumvent α-mannosidase II and the swainsonine block (Romero and Herscovics, 1986; Chui et al., 1997).

Somatic tumor cell mutants with a deficiency in UDP-Gal transport activity show the most severe attenuation of malignancy, suggesting that a blocker of lactosamine extension in N- and O-glycans may be an effective anti-cancer agent. Alternatively, administering a combination of inhibitors of polylactosamine extension and β1,6GlcNAc-branching in the N- and O-glycosylation pathways may prove to be a potent anti-cancer strategy. These strategies will also circumvent side effects associated with carbohydrate storage in lysosomes. Further cancer studies with mutant mice lacking specific glycosyltransferase genes will increase our understanding of the relative importance of these glycan structures to cancer growth and metastasis, and should provide useful information for drug development efforts.

HERITABLE HUMAN GLYCOSYLATION DISORDERS AND ENGINEERED MUTATIONS IN MICE

Somatic glycosylation tumor cell mutants do not have obvious cell-autonomous phenotypes in tissue culture. This is perhaps not surprising, given the predicted role for
glycosylation in cell-cell and cell-ECM interactions. Therefore, analyses of whole organisms are instructive when investigating the role of glycosylation in mammalian biology. The insights into the functions of specific glycosylated structures, provided by rare human heritable disorders and engineered mutations in mice are summarized below.

**Mutations affecting initiation and branching of N-glycans:** The rare human heritable disorder CDGSI (carbohydrate deficient glycoprotein syndrome type I) is characterized by hypo-N-glycosylation of available sites, and clinically presents with severe neurologic dysfunction, multisystemic abnormalities and developmental delay observed in infancy (Keir et al., 1999). The majority of CDGS type I patients are deficient in phosphomannomutase activity, and are therefore depleted of GDP-mannose, a required substrate for synthesis of dolichol-phosphate-mannose, dolichol pyrophosphate oligosaccharide and glycosyl-phosphatidyl-inositol anchors (Hansen et al., 1997; Keir et al., 1999). Consequently, the defect disrupts oligosaccharide transfer to Asn residues and GPI anchor sites of nascent glycoproteins. CDGS type I patients show subnormal mannose levels in serum, and the addition of mannose to cultured cells from CDGS type I patients corrects the hypoglycosylation phenotype (Alton et al., 1999; Panneerselvam et al., 1999). Thus, normalizing blood mannose levels might correct protein hypoglycosylation in some patients, an intervention now being explored (Alton et al., 1999).

GlcNAc-TI action is a prerequisite for the biosynthesis of the entire hybrid and complex-type glycan series (Figure 1), and in mouse appears to be encoded by a single gene, *Mgat1* (Ioffe and Stanley, 1994; Metzler et al., 1994). *Mgat1* message is ubiquitously expressed, as are the complex-type glycan products of the GlcNAc-TI enzyme (Campbell et al., 1995). GlcNAc-TI deficient mouse embryos die at around E9.5 due to a failure of multiple organ systems, including deformation of the neural tube (Ioffe
Complex-type glycans are observed in pre-E6 day \( Mgat1^+ \) embryos, but are absent in E9.5 embryos, suggesting that maternal compensation may prevent even earlier lethality (Ioffe et al., 1997). Lec1, a mutant CHO cell line deficient in GlcNAc-TI, has no observable phenotype in tissue culture (Stanley, 1984), suggesting that GlcNAc-TI activity is not essential for cell-autonomous functions. Consistently, chimeric analysis of embryos has shown that GlcNAc-TI deficient ES cells were able to contribute to every tissue with the exception of bronchial epithelium (Ioffe et al., 1996). This finding also implies that GlcNAc-TI mediated glycosylation does not have an essential cell autonomous role in the majority of tissues, but is required for the differentiation and/or maintenance of bronchial epithelium. Curiously, \( Mgat1^+ \) ES cells also contributed significantly less to bronchial epithelium, suggesting that a full dose of GlcNAc-TI activity is required for the proper formation of this cell layer. The degree to which the null cells in other tissues are functionally normal by virtue of their contact with wild-type cells remains to be determined.

Following the action of GlcNAc-TI, the majority of complex N-glycan structures are processed by \( \alpha \)-mannosidase II. However, an alternate route to complex N-glycans was discovered by analyzing residual glycan structures and activities in the \( \alpha \)-mannosidase II deficient mice (Chui et al., 1997). A new enzyme, \( \alpha \)-mannosidase III, which uses \( \text{Man}_5\text{GlcNAc}_2 \) as substrate (Figure 1), was detected in most tissues of the \( \alpha \)-Mannosidase II deficient mouse, resulting in a partial loss of complex-type N-glycans only. In erythroid cells, \( \alpha \)-mannosidase III activity is not evident, and therefore complex N-glycans are not synthesized. The \( \alpha \)-mannosidase II deficient mice are viable and fertile, but develop a condition similar to human congenital dyserythropoietic anemia (CDA type II), also known as HEMPAS, a genetic disorder characterized by anemia,
splenomegaly and marrow erythroplasia, but without neurological involvement. CDA II lymphocytes and red cells exhibit decreased complex-type glycan content, and in some patients, reduced α-mannosidase II levels were observed. However, the presence of normal α-mannosidase II levels in some patients suggests that the syndrome may have multiple biochemical causes. In patients with α-mannosidase II deficiency, the exact genetic defect remains unidentified, but may involve aberrant transcriptional regulation (Iolascon et al., 1999).

GlcNAc-TII uses the α-mannosidase II-trimmed core as substrate, and its activity is required for the biosynthesis of complex N-glycans (Schachter, 1986). In its absence, hybrid-type structures are formed and only a portion of sialyllactosamine antennae can be added (Figure 1). Like GlcNAc-TI, its activity appears to be ubiquitous. Mice lacking GlcNAc-TII are runted, and die at or shortly after birth with multiple organ defects (Priatel et al., 1997). As is the case with GlcNAc-TI, murine GlcNAc-TII activity appears to be encoded by a single gene. GlcNAc-TII deficiency causes the rare human autosomal recessive disease, carbohydrate-deficient glycoprotein syndrome type II (CDGS type II) (Jaeken et al., 1994). CDGS type II is characterized by multisystemic involvement and severe impairment of the nervous system. Inactivating point mutations in the catalytic domain of the GlcNAc-TII gene (Ser290Phe and His 262Arg) have been identified in two unrelated patients (Tan et al., 1996).

Substitution by GlcNAc-TIII adds a bisecting GlcNAc-residue in a β1,4 linkage to the underlying mannose of the N-glycan core, and renders the glycan chains poor substrates for α-mannosidase II, GlcNAc-TII, GlcNAc-TIV and GlcNAc-TV, thus diverting the pathway towards hybrid glycans as shown in Figure 1 (Schachter, 1986). GlcNAc-TIII activity is normally detected in brain, kidney and B cells (Miyoshi et al.,
Mice deficient in GlcNAc-TIII are viable and fertile with no obvious abnormalities (Priatel et al., 1997; Bhaumik et al., 1998). Although the C. elegans genome has the coding potential for most known glycosyltransferase genes, GlcNAc-TIII appears to have no homologue in the worm (C.E. Warren, personal communication). It is, therefore, likely that GlcNAc-TIII function is specific to vertebrates or mammals, and therefore GlcNAc-TIII deficient mice might respond aberrantly to stimuli of higher organisms, such as stressors of the immune system or carcinogens. Indeed, GlcNAc-TIII deficient mice respond differently to diethynitrosamine than do wild type mice. Exposure to diethynitrosamine results in hepatocarcinogenesis, which is inhibited by GlcNAc-TIII deficiency. However, Mgat3, the gene encoding GlcNAc-TIII, is not expressed in normal or transformed hepatocytes, suggesting that a host factor is dependent on GlcNAc-TIII-dependent glycosylation to promote cancer progression (Bhaumik et al., 1998).

**Mutations affecting O-glycosylation:** To date, few somatic or naturally occurring mutations have been identified that specifically affect O-glycan biosynthesis. In polyagglutination is a notable exception. This is a rare, acquired hematological abnormality affecting a subset of bone marrow stem cells, where a loss of β1,3Gal-T activity due to transcriptional repression is observed (Thurnher et al., 1999) (Figure 2). Core 2 GlcNAc-T initiates β1,6GlcNAc-branched of O-glycans, and its expression is subject to tissue-specific regulation (Granovsky et al., 1995). The antenna initiated by core 2 GlcNAc-T forms the primary scaffold for polylactosamine and SLe^x^ on O-glycans of PSGL-1, and is required for P- and E- selectin binding (Ellies et al., 1998; Li et al., 1996). Polylactosamine on β1,6GlcNAc-branched O-glycans of CD43 and the CD45 on thymocytes bind to the mammalian lectin, galectin-1, found on thymic epithelial cells and this interaction causes enhanced apoptosis (Perillo et al., 1995).
CD43 acts as an anti-adhesive at the leading edge of migrating T cells and facilitates extravasation from the blood into secondary lymphoid tissues (Manjunath et al., 1995; McEvoy et al., 1997). Core 2 GlcNAc-T activity and branched O-glycans on CD43 are upregulated in peripheral T and B cells following activation by antigens, possibly enhancing the anti-adhesive properties of CD43 (Piller et al., 1988). The high density of O-linked glycan chains on CD43 results in an extended protein structure protruding from the cell surface on T lymphocytes, activated B cells, NK cells and immature dendritic cells (Cyster et al., 1991). Transgenic mice expressing Core 2 GlcNAc-T under the regulation of the T-cell specific Lck promoter, display reduced delayed type hypersensitivity reactions in vivo. T-cells from these mice adhere less to fibronectin and ICAM-1 (Tsuboi and Fukuda, 1997).

The gene encoding Core 2 GlcNAc-T has been knocked out in mice. Deficient mice are viable and fertile, with no detectable core 2 GlcNAc-TV enzymatic activity or β1,6GlcNAc-branched O-glycans. The mice develop neutrophilia and partial deficiency of selectin ligands (Ellies et al., 1998). Loss of core 2 oligosaccharides reduces neutrophil rolling in vitro and neutrophil recruitment to sites of inflammation in vivo. However, although core 2 GlcNAc-T deficiency significantly reduces E-, L- and P-selectin ligands on leukocytes, it does not ablate the presence of selectin ligands entirely, suggesting that oligosaccharides other than β1,6GlcNAc-branched O-glycans can be substrates for selectin ligand biosynthesis. It is surprising that β1,6GlcNAc-branching of O-glycans is not detected in the core 2 GlcNAc-T deficient mouse because a second gene encoding an enzyme with core 2, core 4 and “T” catalytic activity has been isolated and characterized (Yeh et al., 1999). Therefore, it is possible that the relatively mild phenotype observed in this mouse is due to compensation by this second gene.
**Mutations affecting Glycan elongation:** Branched N- and O-glycans, as well as glycolipids are elongated by polylactosamine chains (2 to \( >10 \) repeating units of Gal\( \beta 1,4 \)GlcNAc\( \beta 1,3 \)). \( \beta 1,4 \)Gal-Ts substitute GlcNAc\( \beta 1,3 \)-antennae, and their action is essential for polylactosamine production (Figure 1). The predominant mammalian enzyme, \( \beta 1,4 \)Gal-T1 has two distinct functions. In the trans-Golgi network of most cells, the enzyme participates in polylactosamine production, but in lactating mammary gland, \( \beta 1,4 \)Gal-T1 complexes with \( \alpha \)-lactalbumin, altering the enzyme's catalytic specificity to favor glucose over GlcNAc (Bell et al., 1976). Under these circumstances, the enzyme preferentially participates in lactose biosynthesis. \( \beta 1,4 \)GalT1-deficient mice show a marked decrease in galactosylation of serum glycoproteins (Asano et al., 1997). These mice are viable and fertile, but exhibit growth retardation and early lethality. This gross phenotype might be related to the observed abnormalities in epithelial cell proliferation and differentiation of skin and intestine, which suggest that \( \beta 1,4 \)GalT1 is required for the maintenance of the epithelial cell layer after birth.

\( \beta 1,4 \)GalT expressed on the surface of sperm may bind the egg coat glycoprotein ZP3, thus mediating gamete recognition. Indeed, \( \beta 1,4 \)GalT1 deficient sperm bound less ZP3 than wild type sperm, and were unable to undergo acrosome reaction in response to either ZP3 or anti-galactosyltransferase antibody, which rendered them physiologically inferior to wild-type sperm. However, \( \beta 1,4 \)GalT1 null males are fertile, indicating that efficient ZP3 binding and the subsequent induction of the acrosome reaction are not essential for fertilization (Lu and Shur, 1997).

The viability and fertility of \( \beta 1,4 \)GalT1 deficient mice might be attributable to compensation by other enzymes possessing similar activity. To date, 5 additional human \( \beta 1,4 \)Gal-Ts have been identified and cloned with the aid of ESTs. These enzymes,
designated $\beta 1,4$Gal-TII through $\beta 1,4$Gal-TVI, have 33% to 55% sequence identity to $\beta 1,4$Gal-TI (Lo et al., 1998).

**Mutations affecting glycan capping:** The terminal sequences of N- and O-glycans usually contain fucose and/or sialic acid residues. There are 8 mammalian genes encoding fucosyltransferases and at least 13 genes encoding sialyltransferases (reviewed in (Oriol et al., 1999) and (Tsuji, 1996)). Fucose residues are present on N- and O-glycans in one of four possible linkages ($\alpha 1,2$, $\alpha 1,3$, $\alpha 1,4$ and $\alpha 1,6$). Sialyltransferases transfer sialic acids, of which there are 20 isotypes, in four possible linkages with remarkable specificity for the carbohydrate acceptor ($\alpha 2,3$Gal, $\alpha 2,6$Gal, $\alpha 2,6$GalNAc and $\alpha 2,8$Sia) (Tsuji, 1996). As has been previously discussed, genetic and epigenetic diversity of terminal oligosaccharide sequences in the human population may have evolved as a defense against bacterial and viral pathogens, including influenza A, E. coli O157, and Neisseria gonorrhoea, all of which exploit glycoconjugates in their host recognition and subsequent pathogenicity. For example, Influenza A hemagglutinin is known to bind sialic acid in an isotype and linkage-specific manner, which affects the tissue tropism and the infectivity of the virus (Pritchett et al., 1987).

Fucosyltransferases are ancient glycosyltransferases, conserved from prokaryotes to mammals (Oriol et al., 1999). The bacterium Helicobacter pylori is known to synthesize Le$^x$ epitopes, for which $\alpha 1,3$Fuc-T activity is required. The $\alpha 1,3$Fuc-T gene found in another bacterium, Schistosoma mansoni appears to be murine in origin (Oriol et al., 1999). This gene encodes a fucosyltransferase with sialyllactosamine acceptor specificity, an acceptor that is not synthesized by Schistosoma mansoni. It is suggested that the parasite has incorporated a fragment of mouse DNA into its genome. It would be
interesting to know what selective advantage, if any, the bacterium derives from incorporating this mammalian fucosyltransferase into its genome.

In mammals, α1,3Fuc-Ts synthesise the Lewis epitope, SLe^x, on E- and P-selectin ligands. The selectin ligands are a subset of fucosylated O-glycan structures on PSGL-1, ESL-1, GlyCAM-1, CD43 and MadCam (Springer, 1994). Selectin-Selectin ligand interactions enable leukocytes to roll on endothelial cells, a prerequisite for extravasation into sites of inflammation (Vestweber and Blanks, 1999). Five human genes encode α1,3Fuc-Ts (III, IV, V, VI, VII). Genes encoding only 2 of these enzymes, α1,3Fuc-TVII and α1,3Fuc -TIV, are expressed in leukocytes, and α1,3Fuc-TVII appears to be the most active in the synthesis of SLe^x (Knibbs et al., 1996). Mice lacking α1,3Fuc-TVII develop blood leukocytosis due to a deficiency in leukocyte homing and recirculation (Maly et al., 1996). These mice also have poor leukocyte extravasation into areas of inflammation. α1,3Fuc-TVII^-/- mice are phenotypically similar to P- and E-selectin deficient mice and to individuals with LADII. Leukocyte adhesion deficiency II syndrome (LADII) is a rare human congenital disease caused by a defect in GDP-fucose biosynthesis, and is characterized by immunodeficiency and a severe delay in wound healing (Karsan et al., 1998). As discussed above, fucose is essential for the biosynthesis of selectin ligands and is, therefore, important in neutrophil and lymphocyte homing.

In contrast to the severe consequences of α1,3Fuc-TVII deficiency, inactivating mutations in α1,3/4Fuc-TIII are common in the population and impart no obvious phenotype (Orntoft et al., 1996). Similarly, lack of functional α1,2Fuc-TI and TII genes, required for the biosynthesis of ABO and H blood group oligosaccharide antigens, occurs with no evidence of pathology, and inactivating mutations in α1,3FucTVI also cause no apparent harm (Kelly et al., 1994).
Fucosyltransferases and sialyltransferases often compete for the same acceptors. Sialylation is absent in *C. elegans*, which cap its glycans with fucose (Oriol et al., 1999), suggesting that sialylation may be required for functions restricted to vertebrates. The roles ascribed to sialylation include enhanced cellular migration during neurodevelopment and regulating leukocyte extravasation from blood to sites of inflammation and lymphoid organs (Sjoberg et al., 1999). The regulated expression of sialic acids is due in large part to the spatial and temporal pattern of expression of sialyltransferases. These enzymes are grouped into four families according to the carbohydrate linkages they produce; ST3Gal, ST6Gal, ST6GalNAc, and ST8Sia, and are sensitive to the oligosaccharide composition of the acceptor beyond the specific sugar residue to be substituted (Sjoberg et al., 1999). In mammals, ST6Gal sialyltransferase appears to be solely responsible for producing the Siaα2,6Galβ1,4GlcNAc terminus on N-glycans and possibly on some O-glycans (Hennet et al., 1998). The transcription of this gene is regulated by multiple promoters and altered by glucocorticoids and cytokines. ST6Gal is unique in producing the ligand for the CD22 lectin molecule expressed on B cells, a member of the I-type lectin family, which also includes myelin-associated glycoprotein (Sgroi et al., 1993). As CD22 itself carries Sia6LacNAc, homotypic binding interactions have been shown to occur and may play a regulatory role in immune function. Following antigen stimulation of B cells, the cytosolic domain of CD22 recruits the SHP1 tyrosine phosphatase into the Ig receptor-signaling complex, and dampens intracellular signaling. Predictably, therefore, B cells in mice lacking CD22 are hypersensitive to antigens, while ST6Gal deficient mice show impaired B cell maturation and IgM production. The phenotype of ST6Gal deficient mice appears to be more severe than the phenotype resulting from CD22 deficiency, suggesting that there might be other,
as yet unidentified, Sia6LacNAc lectins with regulatory functions in B cells (Hennet et al., 1998).

As described above, whole animal models of aberrant glycosylation provide new insights into the function of specific carbohydrate structures. In particular, immunity and cancer progression and metastasis are complex phenomena, which involve many cellular responses to the cell's environment, such as signal-induced proliferation and/or differentiation, and ECM-mediated cellular adhesion and migration. The complexity of these interactions cannot be fully mimicked in tissue culture, and therefore in vitro models have limited use for studying the role of glycosylation in these processes. In this regard, the development of gene knockout technology has been a timely blessing for the study of biological functions of glycosylation.

**GlcNAc-TV**

GlcNAc-TV is a branching enzyme in the complex-type N-glycosylation biosynthetic pathway (Cummings and Kornfeld, 1984). As such, it adds complexity to the N-glycans it acts upon, by providing the β1,6GlcNAc-branch, an enzymatically favored substrate for the addition of polylactosamine chains. Indeed, GlcNAc-TV activity appears to be rate limiting for polylactosamine addition to N-glycans in tumor cells (Easton et al., 1991). For example, GlcNAc-TV deficiency in MDAY-D2 cells results in significant loss of polylactosamine on N-glycans, but not on O-glycans (Yousefi et al., 1991).

The rat gene for GlcNAc-TV encodes a 740 amino acid (aa) type II transmembrane protein (Shoreibah et al., 1993), a structure shared with all glycosyltransferases cloned to date. Type II transmembrane proteins comprise a short
cytoplasmic N-terminus tail, a single hydrophobic transmembrane domain followed by a proline-rich stem region, and a large catalytic domain at the C terminus. The C-terminal portion of rat GlcNAc-TV (aa 213-740) is essential for catalytic activity (Korczak et al., 1997), and the transmembrane domain and at least one residue in the stem region (aa 188) are required for Golgi localization. Two CHO mutant cell lines, Lec4 and Lec 4A, lack β1,6GlcNAc-branched N-glycans on their cell surface (Weinstein et al., 1996). Lec4 cells do not have GlcNAc-TV activity, due to the disruption of the gene encoding the enzyme, but Lec4A cells have GlcNAc-TV enzymatic activity. The gene encoding GlcNAc-TV in Lec4A cells has a missense mutation, converting leucine to arginine at position 188 (L188R), causing loss of Golgi localization. Therefore, the L188R mutation causes a GlcNAc-TV deficient cellular phenotype by mislocalizing the enzyme without affecting catalytic activity.

GlcNAc-TV is conserved in metazoans. The C.elegans gene, Gly-2, encodes a protein possessing GlcNAc-TV enzymatic activity (Warren and Dennis, 1999, submitted). The gene is able to rescue the cell surface phenotype of Lec4 cells, but not when a L116R mutation, analogous to the L188R mutation in Lec4A, is introduced. Thus, Gly-2 is orthologous to the mammalian genes encoding GlcNAc-TV. It is structurally conserved at both the genomic and peptide levels, and it is functionally interchangeable with mammalian GlcNAc-TV. Such “deep homology” has been demonstrated for only a few genes, the products of which are important within ancient and conserved pathways.

Gly-2 and mammalian genes encoding GlcNAc-TV are multi-exonic, and the structure of the gene (i.e. intron-exon boundries) is conserved between worm and human. The human gene is comprised of 17 exons spanning 155kb and is located on chromosome 2q21 (Saito et al., 1995). Database searches and the characterization of the Mgate^{−/−}
mouse (described in Chapter 3 of this thesis) suggest that mammalian and worm GlcNAc-TV activity is encoded by a single gene. This is an unusual feature, as many glycosyltransferases are encoded by families of genes in *C. elegans* and/or in mammalian species.

GlcNAc-TV gene expression and enzyme activity appear to be regulated in a complex fashion. *Mgat5* gene transcription is regulated by Ras activation (Chen et al., 1998; Dennis et al., 1987). The promoter region of the human gene contains AP1, AP2, HNF1, HP1 and PEA3/ets binding sites (Saito et al., 1995). The v-src oncogene has been shown to stimulate transcription from the *Mgat5* promoter by activating Raf-1 kinase and the Ets-2 transcription factor (Buckhaults et al., 1997). GlcNAc-TV message is detectable in most rat and mouse tissues, albeit in greatly varying amounts (Granovsky et al., 1995; Perng et al., 1994). In addition, levels of GlcNAc-TV mRNA do not necessarily reflect the levels of enzymatic activity in a particular tissue. For example, rat brain has the highest level of GlcNAc-TV transcripts but low levels of enzymatic activity and product, suggesting additional post-translational and/or post-transcriptional regulatory mechanisms (Perng et al., 1994). Intriguingly, transfectants with high expression of GlcNAc-TV do not survive in tissue culture and *Mgat5* transfection enhanced apoptosis, suggesting that there is selection against overexpression of GlcNAc-TV (Demetriou et al., 1995). Thus, proper regulation of GlcNAc-TV activity may be important for cellular viability as well as specific glycoprotein functions.

β1,6GlcNAc-branched N-glycans, as detected by L-PHA lectin immunohistochemistry were detected in the majority of epithelial cell populations, with the exception of breast and colon epithelia in rat and human tissues (Li and Roth, 1997). L-PHA is a plant lectin with specific binding to Galβ1,4GlcNAcβ1,6-(Galβ1,4GlcNAcβ1,2)Manα of tri' and tetraantennary N-glycans (Cummings and
High L-PHA reactivity and GlcNAc-TV activity were detected in the small intestine and in the kidney (Dennis and Laferte, 1989). In addition, activation of human CD4+ and CD8+ thymocytes, which induces high cellular proliferation and migration, results in increased GlcNAc-TV activity and β1,6GlcNAc-branching of N-glycans (Lemaire et al., 1994).

GlcNAc-TV activity and β1,6GlcNAc-branching of N-glycans have been correlated with cancer progression and metastasis. β1,6GlcNAc-branching of N-glycans increases in BHK cells transformed with polyomavirus or RSV virus (Pierce and Arango, 1986; Yamashita et al., 1984). GlcNAc-TV activity increased in NIH3T3 cells transformed with activated Ras, and in rat2 fibroblasts and SP1 epithelial cells transfected with activated Ras or the v-Fps oncogene (Dennis et al., 1989; Dennis et al., 1987; Heffeman et al., 1993; Lu and Chaney, 1993). This enhancement correlated with phenotypic changes consistent with morphological transformation. Increased L-PHA reactivity was reported in a variety of malignant and invasive human tumors, including melanomas and tumors in breast and colon. For example, L-PHA staining was significantly increased in 50% of malignant human breast biopsies and intensity of L-PHA staining significantly correlated with the progression of these malignant tumors (Fernandes et al., 1991). In addition, L-PHA staining in human colorectal carcinoma sections provides an independent prognostic indicator for tumor recurrence and patient outcome, and is associated with the presence of lymph node metastases (Seelentag et al., 1998).

There is evidence that the relationship between increased β1,6 branching of N-glycans and aspects of the malignant phenotype is causal. Somatic mutant tumor cells selected for reduced L-PHA reactivity and GlcNAc-TV activity are compromised in their ability to form lung colonies post i.v. injection, while cell lines with increased sensitivity
to L-PHA show enhanced metastatic potential (Dennis et al., 1986; Dennis et al., 1987). In addition, N-glycosylation inhibitors, such as swainsonine, are able to slow solid tumor growth in mice and affect proliferation of murine lymphoma and human sarcoma lines in serum free medium (Goss et al., 1995). Two genetic studies looked at the specific role of GlcNAc-TV in transformation and metastasis. Demetriou et al. transfected rat GlcNAc-TV cDNA into Mv1Lu cells, a premalignant immortalized mink lung epithelial cell line (Demetriou et al., 1995). The forced expression of GlcNAc-TV and the subsequent increase in β1,6-branched N-glycans resulted in loss of contact-inhibition of cell growth. GlcNAc-TV transfectants formed foci in serum-deprived and high density monolayer cultures, and maintained microfilament structures consistent with a proliferative state. These phenotypic traits correlated with the degree of GlcNAc-TV expression. In vivo, transfectants with the highest levels of GlcNAc-TV expression and activity were able to form solid tumors in nude mice (Demetriou et al., 1995).

More recently, Seberger and Chaney studied metastatic and transformed cell lines transfected with GlcNAc-TV cDNA (Seberger and Chaney, 1999). The transfection resulted in an increase in the metastatic potential of these cells by 4-40 fold. The increased metastatic potential was not associated with a gross change in cellular growth rate as measured in tissue culture, nor with aberrant adhesion to extracellular matrix proteins, such as fibronectin or collagen type IV.

In order to understand the role of GlcNAc-TV in mammalian development and in cancer progression and metastasis, we undertook to study its expression pattern during murine embryogenesis and to generate GlcNAc-TV deficient mice. We have confirmed that GlcNAc-TV is not ubiquitously expressed, and that its expression is highest in cells with stem-cell-like properties, i.e. possessing regenerative and migratory capacity. The knockout mouse has provided us with a possible explanation of how β1,6GlcNAc-
branched N-glycans participate in such processes as T cell and tumor cell proliferation, and leukocyte adhesion and migration. The data suggest a direct regulatory role for GlcNAc-TV in T cell receptor and integrin transmembrane receptor aggregation and subsequent signaling.
The cDNA encoding rat GlcNAc-TV was cloned shortly before I started my studies. We were, therefore, in a position to take a genetic approach to study the function of \( \beta 1,6 \text{GlcNAc-branched N-glycans} \).

I first determined the temporal and spatial expression pattern of \( Mgat5 \), the gene encoding GlcNAc-TV, during murine embryogenesis. High \( Mgat5 \) expression was observed in the giant cells of the trophoblast, basal epithelia in skin and intestine and in the cortical neural epithelium. A common feature of these cells is their capacity to migrate, a cellular function that may require GlcNAc-TV-dependent glycosylation. The detailed expression pattern is described in Chapter 2.

To study the role of GlcNAc-TV in mammalian biology, we generated GlcNAc-TV deficient mice by targeting the \( Mgat5 \) locus by homologous recombination in embryonic stem (ES) cells. Chapter 3 describes these mice, with particular emphasis on the function of leukocytes and T-lymphocytes. We hypothesized that if \( \beta 1,6 \text{GlcNAc-branched N-glycans} \) regulate cellular migration, leukocyte migration in response to inflammatory stimuli would be altered by GlcNAc-TV deficiency. Two independent models of acute inflammation were used to test this hypothesis. In addition, a delayed type hypersensitivity model was used to study the effect of GlcNAc-TV deficiency on T cell function \textit{in vivo}. \( Mgat5^+ \) mice responded more strongly to the delayed type hypersensitivity antigen than did wild type mice. This result prompted an \textit{in vitro} study of \( Mgat5^+ \) T cell response to mitogens.

We hypothesized that tumor progression and metastasis may be impeded in GlcNAc-TV deficient mice. This hypothesis was based on work with GlcNAc-TV deficient somatic tumor cell mutants done previously in the Dennis lab and in several other labs, consistently showing slower tumor progression and reduced metastasis. In addition, studies
with MvLu1 cells and mammary carcinoma cell lines transfected to overexpress GlcNAc-TV, suggest that GlcNAc-TV activity may be required for transformation and metastasis. Since all previous studies have been done with transplanted tumor cells, which were already fully transformed, the role of GlcNAc-TV in tumor initiation, or the effect of GlcNAc-TV deficiency in the host animal on tumor progression could not be studied. Chapter 4 describes the effect of GlcNAc-TV on tumor formation and development in a whole animal model in which GlcNAc-TV deficient mice were crossed with transgenic mice expressing the polyoma middle T oncogene under the control of a mammary-specific promoter (MMTV-PyMT). PyMT is a potent oncogene, and its transforming activity is dependent on its ability to associate and activate c-Src, PI3K and Shc. Expression of the PyMT transgene in mammary epithelium results in global hyperplasia, multifocal mammary adenocarcinomas and frequent metastases to the lung (Guy et al., 1992). Therefore, this cross gave us the opportunity to study the effect of GlcNAc-TV on tumor formation, tumor growth, metastatic potential and oncogenic pathways dependent on GlcNAc-TV mediated glycosylation.
CHAPTER 2: GlcNAc-TV and Core 2 GlcNAc-T Expression in the Developing Mouse Embryo.

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UDP-GlcNAc:Manα1,6Manβ-R β1,6-N-acetylglucosaminyltransferase V (GlcNAc-TV) and UDP-GlcNAc:Galβ1,3GalNAc-R β1,6-N-acetylglucosaminyltransferase (core 2 GlcNAc-T) are Golgi enzymes that catalyze the biosynthesis of β1,6GlcNAc-branched intermediates in the N- and O-linked biosynthesis pathways, respectively. The activities of these enzymes change during haematopoiesis, embryocarcinoma cell differentiation and following malignant transformation, but little is known about their expression in normal adult tissues and during embryogenesis. We have examined the expression of GlcNAc-TV and core 2 GlcNAc-T in sections of post-implantation mouse embryos by in situ RNA hybridization. The two enzymes showed distinct temporal and spatial patterns of expression. Core 2 GlcNAc-T mRNA was widely expressed at embryonic day (E) 7 and became restricted to a subset of mucin-producing and cartilagenous tissues at E11.5 through to E17.5. GlcNAc-TV transcripts were absent at E7, became expressed throughout E9.5 embryos, and then progressively restricted to regions of the developing central nervous system (CNS) and to specialized epithelia of skin, intestine, kidney, endocrine tissues and respiratory tract. In the adult gonads, GlcNAc-TV transcripts were excluded from germ cells but were detected in the follicular and testicular cells. Leukoagglutinin (L-PHA)- reactive oligosaccharides co-localized with GlcNAc-TV transcripts in skin, kidney and intestine, but brain showed unexpectedly low overall staining punctuated by bright staining of the vascular endothelium. A common feature of cells in basal epithelia and in the cortical neural epithelium is the capacity to migrate, a cellular function which may require GlcNAc-TV-dependent glycoconjugates.
INTRODUCTION

Oligosaccharides, such as the stage-specific embryonic antigens (eg. Lewis antigens), show precise spatial and temporal patterns of expression during mouse embryogenesis (Childs et al., 1983; Feizi, 1985; Hakomori and Kannagi, 1983; Pennington et al., 1985; Solter and Knowles, 1978). These carbohydrate sequences form the termini of N- and O-linked oligosaccharides and can serve as ligands for lectins, or may directly mediate cell-cell interactions by homotypic binding (Eggens et al., 1989; Fenderson et al., 1990). Multivalent conjugates of Le' have been shown to inhibit mouse embryo compaction in vitro, suggesting that they may participate in cell-cell interactions at this early stage (Bird and Kimber, 1984; Fenderson et al., 1984). In adult tissues, sialylLe' sequences are expressed on neutrophil cell surface glycoproteins which can mediate cell adhesion by binding to selectins found on activated or damaged vascular endothelia (Springer, 1994). Similar interactions may be involved in cancer cell invasion and metastasis, as these embryonic carbohydrate sequences are commonly re-expressed in cancer cells (Dennis, 1991).

Galβ1,3GalNAcα-Ser/Thr and Manα1,3[Manα1,6]Manβ1-4GlcNAcβ1,2GlcNAc-Asn, common core structures for O- and N- linked glycans respectively, are substituted to varying degrees by branching GlcNAc-Ts in the medial Golgi. This allows for considerable structural diversity as the GlcNAc residues can be extended by the many glycosyltransferases of the trans-Golgi to produce different terminal sequences. Acceptor specificity and competition between glycosyltransferases for common acceptors (Schachter, 1986), coupled with tissue-specific expression of the enzymes, appear to be major factors regulating the distribution of oligosaccharide structures (Paulson and Colley, 1989a; Paulson et al., 1989b). GlcNAc-TV substitutes GlcNAcβ1,2Manα1,6Manβ- of the core N-linked structure at the 6 position of the α1,6 linked mannose, creating a preferred acceptor for
polylactosamine extension and associated Lewis antigens (van den Eijnden et al., 1988; Yousefi et al., 1991). In the O-linked oligosaccharide pathway, core 2 GlcNAc-T plays a similar role, converting core 1 (ie. Galβ1,3GlcNAcα-O) to core 2 (ie. Galβ1,3(GlcNAcβ1,6)GalNAcα-O), thereby creating a branch for the addition of polylactosamine (Carlsson et al., 1986; Yousefi et al., 1991). Oncogene-induced transformation of fibroblasts (Dennis et al., 1987; Lu and Chaney, 1993; Pierce and Arango, 1986; Yamashita et al., 1985), differentiation of teratocarcinoma cells (Heffernan et al., 1993), and activation of lymphocytes (Lemaire et al., 1994) have been shown to induce GlcNAc-TV activity and N-glycan-associated polylactosamine. Core 2 GlcNAc-T activity and O-glycan associated polylactosamine is also elevated following lymphocyte activation (Higgins et al., 1991; Piller et al., 1988) and in some malignant cells (Higgins et al., 1991). Therefore, GlcNAc-TV and core 2 GlcNAc-T activities are developmentally-regulated, and these enzymes control expression of polylactosamine and associated terminal carbohydrate sequences in N- and O- linked glycan biosynthesis pathways.

To initiate a genetic study of the role of GlcNAc-transferases in mouse development, we have examined the tissue distribution of core 2 GlcNAc-T and GlcNAc-TV transcripts during mouse embryogenesis by in situ RNA hybridization. GlcNAc-TV and core 2 GlcNAc-T showed distinctive temporal and spatial patterns of expression in post-implantation embryos. Core 2 GlcNAc-T was expressed at E7 prior to organogenesis and became restricted to mucin-producing and cartilaginous tissues in later-stage embryos. GlcNAc-TV was upregulated in a widespread manner in E9.5 embryos, and later became confined to several specialized epithelial cell layers, as well as cells found in the neuroepithelium of the developing CNS.
RESULTS

GlcNAc-TV expression in post-implantation mouse embryo: Implantation of blastocyst stage embryos into the uterine epithelium takes place between E4.5 and E6, and by E7 in primitive streak embryos, the three embryonic cell layers can be readily be identified in sagittal sections. GlcNAc-TV transcripts were highly expressed in the giant cells of the trophoblast and in the decidua basalis at E7 but absent in the embryo (Figure 1). To compare expression of GlcNAc-TV and its products, lectin histochemistry was also preformed on embryo sections. β1,6GlcNAc-branched oligosaccharides were detected with L-PHA (Cummings and Kornfeld, 1982), which showed very low reactivity on all three cell layers of the E7 embryo when compared to the deciduum which stained heavily (Figure 2). However, ConA and E-PHA stained the E7 embryo intensely, suggesting that biantennary and/or high mannose -type as well as complex-type N-linked oligosaccharides were present, while structures with β1,6GlcNAc-branching were absent. GlcNAc-TV transcript levels were greatly increased and widely distributed in the E9.5 embryo, with particularly high signals observed in the developing neuroepithelium of the CNS (Figure 1). In E9.5 and E10.5 embryos, transcripts were distributed throughout the ventricular zones of the CNS, the region where the actively dividing neuroblasts are located. By E11.5, the accumulation of GlcNAc-TV message shifted to the intermediate and marginal zones of the spinal cord, the cranial flexure, the lamina terminalis and the fourth ventricle. Later stage embryos consistently expressed high levels of message in the marginal and intermediate zones of the CNS which are the more differentiated areas of the nervous system compared to the underlying ventricular zone.
Figure 1: In situ localization of GlcNAc-TV (TV) and core 2 GlcNAc-T (C2) expression during murine embryogenesis.

Bright- and darkfield photomicrographs of sagittal sections through embryos at E7, E9.5, E13.5 and E17.5 as indicated at the left. Tissue sections were prepared and hybridized with antisense RNA probes as described in Materials and Methods. Labels are: ac, amniotic cavity; ex, extraembryonic region; e, ectoplacental cone; fb, forebrain; gu, gut; h, heart; hb, hindbrain; k, kidney; li, liver; lu, lung; mb, midbrain; ne, neuroepithelium; ot, otic vesicle; rp Rathke’s pouch; sc, spinal cord; t, trophoblast; v, vertebrae; wf, whisker follicles; iii 3rd ventricle; iv, 4th ventricle; ba bronchial arches; tr, thyroid; th, thymus; p, pancreas; o, olfactory epithelium.
Figure 2: **Lectin histochemistry of the E7 embryo.**

E7 embryos were stained with (A) E-PHA, (B) L-PHA and (C) ConA. Labels are: am, mesoderm and ectoderm component of amnion; Pe, parietal endoderm; ee extra-embryonic endoderm; m, mesoderm; ne, neural ectoderm.
Expression was also observed in the auditory semicircular canal, in the lens epithelium of the eye and in the olfactory epithelium. Although GlcNAc-TV transcripts appeared to be widely expressed in the central nervous system, L-PHA staining of E15.5 and E17.5 embryos was restricted largely to the vascular endothelium of brain (data not shown).

GlcNAc-TV transcripts were observed in several endocrine organs at different stages of development (Figure 1). At E11.5, expression was observed in the Rathke's pocket, the primordial structure for the pituitary gland, a pattern that persisted through E17.5, when highest expression was detected in the pars distalis of the pituitary gland. In contrast, GlcNAc-TV message was detected in the parathyroid, the adrenal gland and pineal gland only later in organ development as observed in E17.5 sections. The most striking example was the genesis of the parathyroid, which detaches from the thyroid gland at E12, with continued morphological development until E15 when GlcNAc-TV message becomes highly expressed. However, expression was not observed in the thyroid at any of the stages examined. Embryonic thymus showed low GlcNAc-TV expression, although message has previously been detected in adult thymus (Perng et al., 1994). It is possible that GlcNAc-TV expression is upregulated and required in thymocytes after birth, as GlcNAc-TV enzyme activity has been observed to increase following thymocyte activation (Lemaire et al., 1994).

In the gastrointestinal tract, GlcNAc-TV transcripts were observed in embryos at E11.5, the earliest stages of intestinal development, persisting through to E17.5. Furthermore, this pattern of expression was similar in E17.5 embryos and adult intestine (Figure 3B,F). GlcNAc-TV was present in epithelial and Paneth cells at the base of the crypts, and in the columnar epithelial cells in the lower half of the villi.
Figure 3:  GlcNAc-TV and core 2 GlcNAc-T expression in adult and embryonic mouse intestine.

Panels A-D are photomicrographs of sections from E17.5, and panels E,F show adult intestine. Light-fields (A) is paired with the dark field in situ (B), GlcNAc-TV; and (D), core 2 GlcNAc-T. Panel C shows L-PHA staining for GlcNAc-TV product in E17.5 intestine. Panel E is the light-field of a section through adult intestine and it is paired with the dark-field of the same section shown in panel F. Labels are: vi, villi; cr, crypt cells; il, intestinal lumen; sm, smooth muscle layer.
Transcripts were absent in lamina propria and in the smooth muscle layer located immediately below the crypts. L-PHA lectin histochemistry showed reactivity in the epithelium lining the villi, coincident with the location of GlcNAc-TV transcripts (Figure 3C). L-PHA staining extended to the top of the villi, presumably reflecting a longer half-life of the N-linked oligosaccharides than the GlcNAc-TV transcripts in the epithelial cells moving up the villi from the crypts.

GlcNAc-TV was highly expressed in the developing kidney beginning at E13.5, and appeared to be limited to the epithelial cells of Bowman’s capsules, a cell layer which also stained intensely with L-PHA (Figure 4).

In skin, GlcNAc-TV was expressed in the basal cell layer of the epidermis beginning at E15.5 (Figure 5). The basal or peridermal layer of the epidermis is the outermost layer at E14, the earliest stage of skin differentiation, and stratification begins on E16.5. At E15.5, prior to stratification of the epidermis, GlcNAc-TV transcripts and L-PHA reactivity were observed in the outermost cell layer. Following stratification at E17.5, transcripts and L-PHA reactivity were confined to the basal cell layer and absent in the cornified layer. GlcNAc-TV expression was also observed in the whisker follicles at E13.5 and was maintained through E17.5 (Figure 6). Expression was restricted to cells of the external root sheath, an involuted basal epithelium of the skin which also stained strongly with L-PHA (data not shown).

Low levels of GlcNAc-TV message were observed in the nasal epithelium, in the lining of the oral cavity and in the oesophagus and trachea at E11.5. By E15.5, prominent expression was seen in the respiratory epithelium of the nasal cavity, and in the similarly structured epithelium of the pharynx, the oesophagus, the trachea and the bronchi (data not shown). This pattern of expression persisted to E17.5 (Figure 1).
Sertoli and granulosa cells are support cells in the adult testis and ovaries, respectively, both of which expressed GlcNAc-TV message. Transcripts were observed in granulosa cells of superovulated ovaries at all stages of follicle maturation, and in the epithelium of the oviduct (Figure 7). However, message was not detected in spermatozoa or ova.

*Core 2 GlcNAc-T expression in the post-implantation mouse embryo:* Core 2 GlcNAc-T transcripts were highly expressed in embryonic and extraembryonic regions of the E7 embryo, as well as in the giant cells of the trophoblast and the maternal deciduums (Figure 1). The pattern of expression became more restricted by E9.5. Between E9.5 and E11.5, message was detected primarily in tissues of mesodermal origin, such as the liver bud, the visceral arches, the heart primordium and the somites. The expression pattern of core 2 GlcNAc-T in later stage embryos was associated with mucin- and cartilage-producing tissues.

Core 2 GlcNAc-T was not expressed in the oral and nasal epithelium prior to E11.5, but were detected by E13.5 and persisted through E17.5. At E15.5 and E17.5, core 2 GlcNAc-T expression was also detected in the respiratory epithelium of the nasal cavity, the trachea and the pleural sac (Figure 1).

At E9.5, core 2 GlcNAc-T transcripts were highly expressed in the mesenchyme of the somites and in the otic vesicle, regions which will later form cartilage. By E13.5, high expression of core 2 GlcNAc-T message was also observed in the cartilage of the front and hind paws, where cartilage synthesis is a preliminary step to bone formation (data not shown). At E17.5, transcripts were reduced in the paws correlating with reduced cartilage.
Figure 4: GlcNAc-TV and core 2 GlcNAc-T expression in kidney at E17.5.

Light-field photomicrograph (A) is paired with dark-field of *in situ* for core 2 GlcNAc-T (B), and GlcNAc-TV (C). Panel D shows L-PHA staining for GlcNAc-TV product which is localized to Bowmann's capsule, (bc). Other labels are: dt, distal tubule; pt, proximal tubule; gl, glomerulus.
Figure 5: GlcNAc-TV expression in murine skin.

(A), Light-field photomicrograph at E17.5; and corresponding dark-fields of (B), *in situ* for GlcNAc-TV transcript and (C), L-PHA staining. (D), shows in situ for GlcNAc-TV transcript at E15.5. The peridermal layer, which expresses GlcNAc-TV at E15.5 becomes the basal cell layer by day 17.5. Labels are: bc, basal cells; cl, cornified layer; pd, peridermal layer, dm, dermis.
Figure 6: GlcNAc-TV and Core 2GlcNAc-T are not expressed in the same cell populations.

Bright- and corresponding darkfield photomicrographs showing GlcNAc-TV, (A,B); and core 2 GlcNAc-T, (C,D) transcript expression in whisker follicles, and GlcNAc-TV in lung (E,F) at E17.5. Labels are: irs, inner root sheath; ers, external root sheath; cts, connective tissue sheath; rb, respiratory bronchioles.
Figure 7: GlcNAc-TV and core 2 GlcNAc-T expression in female reproductive organs.

*In situ* localization of GlcNAc-TV (B,E) and core 2 GlcNAc-T (C,F) transcripts in superovulated ovary (A-C) and oviduct (D-F). Labels are: fo, follicles; am, amplulla; is, isthmus.
production and higher bone formation at this developmental stage. Other cartilage producing tissues also expressed core 2 GlcNAc-T transcripts at E17.5, including the nasal passages, trachea, invertebrate disks and Meckel's cartilage of the lower jaw (Figure 1). Core 2 GlcNAc-T transcripts were observed in the proximal tubules, the mucin producing compartments of the kidney (Figure 4B). The distal tubules and Bowman's capsule produce less mucin and these regions showed very little message. In the intestine, expression was observed in the epithelial cells of the villi at E11.5, and appeared to increased by E13.5. Expression persisted through E17.5 and in the adult intestine (Figure 3D).

In whisker follicles, core 2 GlcNAc-T message was observed in the inner root sheath, a cell layer close to the lumen which becomes occupied by a hair at E13.5 through E17.5 (Figure 6C,D). This contrasts with GlcNAc-TV transcripts which were restricted to cells of the external root sheath, a cell layer contiguous with the basal cell layer of skin.

Core 2 GlcNAc-T message was not detected in the ovaries of superovulated females. However, the isthmus of the oviducts showed high levels of expression, again correlating with a region of high mucin production (Figure 7). In the adult testis, core 2 GlcNAc-T transcripts were detected in the male germ cells at all stages of spermatogenesis (data not shown).
DISCUSSION

In this study, we have examined the distribution of GlcNAc-TV and core 2 GlcNAc-T transcripts in post implantation mouse embryos by RNA in situ hybridization. Core 2 GlcNAc-T message was expressed widely at E7, considerably earlier than GlcNAc-TV transcripts, which were observed at E9.5 in all embryonic and extraembryonic cell layers. Retinoic acid-induced differentiation of F9 cells into primitive endoderm is believed to reflect a similar developmental period and is accompanied by the induction of several glycosyltransferases (Heffernan et al., 1993). In this system, core 2 GlcNAc-T shows a larger and more rapid induction following retinoic acid treatment than does GlcNAc-TV (Heffernan et al., 1993), a developmental sequence which may be analogous to the earlier appearance of core 2 GlcNAc-T as compared to GlcNAc-TV during embryogenesis. These observations may reflect a general temporal difference in the induction of enzymes in the O-linked and N-linked pathways. Core 2 GlcNAc-T expression becomes restricted to specific tissues by E9.5, and similarly, a restricted expression pattern was observed for GlcNAc-TV by E11.5. However, patterns of tissue-specific expression were distinctly different for the two genes (Figure 8).

Core 2 GlcNAc-T expression was observed in epithelial cells of most, but not all, mucin producing organs, where O-glycosylation is expected to be high. Core 2 GlcNAc-T expression overlapped with a subset of tissues that express the MUCs, with highest levels in the proximal tubules of the kidney, the pancreas, respiratory tract and small intestine (Braga et al., 1992). Core 2 GlcNAc-T transcripts were also observed in the pericardium, the visceral pleura and around the vertebrae, where lubricative and synovial fluid secretions occur. In the adult oviduct, core 2 GlcNAc-T transcripts were highest in the isthmus where mucins are also highly expressed. Core 2-GlcNAc-T transcripts were also found in regions
Figure 8: Summary of the expression patterns.

The temporal and spatial expression pattern of GlcNAc-TV (open box) and core 2 GlcNAc-T (closed box) in the post implantation mouse embryo.
Brain
  ventricular
  medial, marginal

Endocrine
  pituitary
  adrenal
  pineal

Intestine
  villi
  crypts

Kidney
  Proximal tubules
  Bowman’s capsule

Skin

Pancreas

Respiratory epithelium

Cartilage
of cartilage production, suggesting a possible role in bone formation. Core 2 GlcNAc-T expression was not detected in embryonic thymus but the message is present in the cortex of postnatal adult thymus (Baum et al., 1995). Core 2 GlcNAc-T activity has also been shown to increase following activation of human T cells in tissue culture (Higgins et al., 1991; Piller et al., 1988), and may therefore be associated with later stages of T cell maturation.

In the early post-implantation embryo, GlcNAc-TV transcripts show similar temporal regulation as that reported for GlcNAc-TI, with very low levels at E7, followed by up-regulation at E9.5. However, GlcNAc-TI remains widely expressed in all tissues post E9.5 (unpublished data), while GlcNAc-TV becomes progressively restricted to specific cell layers (Campbell et al., 1995). Lectin histochemistry showed that E-PHA reactive complex-type glycans were present in the E7 day embryo, but L-PHA reactive β1,6GlcNAc-branched structures were relatively low. These observations suggest that the oligosaccharide structures are either not necessary, or maternal sources of GlcNAc-TV and GlcNAc-TI and/or glycoconjugates are sufficient for gastrulation and implantation. In the sea urchin embryo, gastrulation occurs in the presence of N-linked processing inhibitors 1-deoxynojirimycin and 1-deoxymannojirimycin but subsequently, spicule formation is blocked suggesting a need for complex-type oligosaccharides at this latter stage of development (Kabakoff and Lennarz, 1990).

E9.5 marks the beginning of rapid embryo growth with a 330x increase in weight by E17.5. Maternal sources of message and proteins are expected to be depleted by dilution during this phase, and therefore autonomous expression of genes becomes essential for rapid growth and continued organogenesis. The induction of GlcNAc-TI, as well as GlcNAc-TV, core 2 GlcNAc-T, β1,3Gal-T, β1-4Gal-T and GlcNAc-T(i) activities observed in retinoic acid treated F9 cells suggests that a general increase in glycoprotein glycosylation occurs following E7, possibly to accommodate the requirements for increased extracellular matrix
and connective tissue glycoproteins. In this regard, GlcNAc-TI null mutant embryos, which are defective in neural tube closure and vascularization, show evidence of abnormal development only after E8.5-E9.5, coincident with the advent of GlcNAc-TI and GlcNAc-TV expression in wild-type embryos (Joffe and Stanley, 1994; Metzler et al., 1994). Because the product of GlcNAc-TI is required in the synthesis of acceptors for GlcNAc-TII, TIV and TV enzymes (Schachter, 1986), its absence results in a complete loss of complex-type N-linked oligosaccharides. Thus, it cannot yet be determined which defects are attributable to the failure to form subsets of structures downstream of GlcNAc-TI. Patients with carbohydrate deficiency syndrome type II, a rare human congenital defect in GlcNAc-TII, show severe multi-organ defects (Jaeken et al., 1994). A point mutation in the coding sequence of the GlcNAc-TII gene from two patients has recently been identified (Schachter et al. personal communication). Further studies with GlcNAc-T gene "knockout" mice will be required to dissect the null phenotypes of GlcNAc-TII and TV, specifically.

As organogenesis proceeds at E9.5 and thereafter, GlcNAc-TV transcript in non-neural tissues becomes progressively restricted to specialized epithelia. The complex pattern of expression of GlcNAc-TV, and its potential to act on many target glycoproteins, suggests that the gene product might be required in a variety of biological functions. A subset of cells which express GlcNAc-TV share the common features of basal cell layers such as their stem cell properties, and attachment to basal lamina. These include basal epithelia of the intestine, skin, lung, and Bowman's capsules in kidney, as well as the Sertoli cells of the testis and the granulosa cells of the ovary. The skin, and gastrointestinal tract rapidly repair denuded epithelium by migration and proliferation of surrounding epithelial cells to cover exposed basement membrane. In the small intestine, this involves proliferation and upward migration of epithelial cells from the lower half of the villus followed by terminal differentiation as the cells continue to ascend to the top portion of the
Although GlcNAc-TV transcripts were absent in the upper portion of the villus, L-PHA staining indicated that the cells retain the oligosaccharide structures formed by the enzyme until the cell are shed.

In normal basal epithelium, cell proliferation and migration is suppressed, but can be rapidly induced in response to cues such as tissue damage. However, the growth and motility of transformed cells are not well controlled by their tissue environment. Transformed fibroblasts and epithelial cell have been shown to have elevated GlcNAc-TV activity (Dennis et al., 1987; Lu and Chaney, 1993; Pierce and Arango, 1986; Yamashita et al., 1985) which may contribute to cell growth and motility. Indeed, transfection of Mv1Lu epithelial cells with a GlcNAc-TV expression vector resulted in relaxation of growth controls and increased cell motility (Demetriou et al., 1995). Trophoblasts, an invasive cell in the E7 embryo also express high levels of both GlcNAc-TV transcripts and L-PHA reactive oligosaccharides. Furthermore, swainsonine, an α-mannosidase II inhibitor that blocks N-linked processing pathway prior to GlcNAc-TV, has been shown to inhibit both tumor cell and first trimester human trophoblast cells invasion in vitro (Yagel et al., 1989; Yagel et al., 1990).

A more recent survey of normal human and rat tissues detected L-PHA reactive glycans in most non-mitotic and non-migrating epithelial cell types, with the notable exception of colonic and mammary gland epithelia (Li and Roth, 1997). Therefore, it is possible that in addition to the suggested role for β1,6GlcNAc-branched N-glycans in aiding cell migration, these glycans have additional functions, not readily apparent from their location. For example, our RNA in situ results indicate that GlcNAc-TV activity may also be necessary for either the formation of secretory epithelia of some endocrine glands, or the proper glycosylation of the hormones that these glands produce. These include the secretory epithelia of the pituitary gland, the parathyroid, the pineal gland and the adrenal gland.
GlcNAc-TV transcript levels appeared to be highest in embryonic neuroepithelium of the brain and spinal cord from E13.5 to E17.5. Expression was first observed at E9.5 to E10.5 in the ventricular zone. In later stage embryos (ie. E11.5-17.5), transcripts were observed in the intermediate and marginal zones, where post mitotic neurons and glial cells are actively migrating outward to populate the cortical layers. Therefore, GlcNAc-TV mRNA expression correlates with regions of active cell migration and neurite outgrowth in the developing CNS. Cerebellar organization also involves selective cell death by apoptosis which may determine the correct size of sensory neural areas and that of its targets. Interestingly, Mv1Lu epithelial cells transfected with a GlcNAc-TV expression vector also show accelerated rates of cell death in serum-free medium (Demetriou et al., 1995), similar to primary mouse fibroblasts partially-transformed by a single oncogene (eg. H-ras) (Tanaka et al., 1994).

Our RNA in situ hybridization and L-PHA histochemistry, as well as previously reported Northern and enzyme activity analysis (Dennis and Laferte, 1989; Pemg et al., 1994) suggest that the ratio of GlcNAc-TV transcripts to enzyme activity in brain is considerably greater than in other GlcNAc-TV expressing tissues such as the intestine and kidney. It is possible that GlcNAc-TV enzyme activity is regulated by post-transcriptional or post-translational mechanism(s) that suppress activity in the central nervous system. For example, β1-4Gal-T activity during testicular development appears to be regulated by alternate splicing of 5' untranslated sequences that affect translation efficiency. In addition, β1-4Gal-T (Bunnell et al., 1990) and core 2 GlcNAc-T (Datti and Dennis, 1993) may also be regulated by protein phosphorylation. The brain GlcNAc-TV transcript is 7.5 kb, similar in size to that of other tissues. However, the GlcNAc-TV gene has multiple exons (Dennis et al. unpublished data), raising the possibility that transcripts might undergo tissue-specific alternative splicing to produce different protein products, as has been observed for α2-6SA-
T (Svensson et al., 1990). Secondly, the predicted protein sequence is 740 amino acids, almost twice the length of the other GlcNAc-Ts suggesting that the enzyme may have additional functional domains not involved in glycosyltransfer.
MATERIALS AND METHODS

Animals: CD1 female mice were mated overnight, checked for vaginal plugs the following morning and gestation time counted from the preceding night. Super-ovulation was achieved by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin, followed 48h later by an injection of 5 IU human chorionic gonadotropin (hCG).

In situ RNA Analysis: cDNA encoding mouse GlcNAc-TV (Shoreibah et al., 1993) and mouse core 2 GlcNAc-T (Warren et al. in preparation, accession #U19265), which is highly homologous to the human gene (Bierhuizen and Fukuda, 1992), were used to prepare the anti-sense RNA probes. The 2384bp HinDIII: BglII fragment of pcDlnGnT-V3 was subcloned into HinDIII: BamHI cut pBluescript KS(+). This plasmid was linearized by HinDIII digestion and anti-sense probes generated by in vitro transcription with T7 RNA polymerase incorporating [γ-35S]UTP. The 1715bp XhoI fragment of pmC2-251 was subcloned into SalI cut pGEM-5zf(+). This construct was linearized by NotI digestion and antisense probe synthesized by in vitro transcription with SP6 RNA polymerase incorporating [γ-35S]UTP.

In situ RNA hybridization was carried out on 7 μm cryostat sections, as previously described (Peter et al., 1990). Adjacent sections were probed with antisense GlcNAc-TV and core 2 GlcNAc-T, and as a control with sense probes. Post-hybridization washes included treatment with 50 μg/ml RNase A at 37°C or 42°C for 30 minutes, and 2 stringent washes of 20 minutes each at 60°C in 0.1X standard saline citrate (15 mM NaCl, 1.5 mM sodium citrate pH 7). The slides were dipped into Kodak NTB-2 emulsion, exposed for 7 days, developed and stained with toluidine blue.

Lectin histochemistry: Cryostat sections of 7 μm in thickness were blocked with 1% BSA in PBS at 20°C for 30 min. FITC-conjugate L-PHA at 20 μg/ml was overlayed onto
the section, and incubated for 30 min at 37°C in a humidified chamber. The slides were then washed twice with PBS, 1% BSA for 10 min each at room temperature. Fluorescence was observed and photographed using a Leica Leitz DM RXE microscope.

In Figure 2, cryostat sections were stained with biotinylated E-PHA and L-PHA (Vector), postfixed for 30 min in 3.7% formaldehyde. The slides were washed in PBS, rinsed in methanol and incubated with 0.3% H₂O₂ in methanol for 20 min. The slides were blocked with 1% BSA in PBS and then incubated with the lectins at 10 ug/ml in the same buffer overnight at room temp. After three washes in PBS, the color was developed with a solution of 1 mg/ml of diaminobenzidine, 0.003% H₂O₂ for 10 min, after which slides were washed in water, mounted under Gel-mount (Biomed) and photographed (Campbell et al., 1995).
CHAPTER 3: Generation and Characterization of GlcNAc-TV Deficient Mice
INTRODUCTION

Tissue-specific patterns of glycoprotein glycosylation (Childs et al., 1983; Feizi, 1985; Hakomori and Kannagi, 1983; Pennington et al., 1985; Solter and Knowles, 1978), and more specifically GlcNAc-TV-dependent glycosylation (Chapter 2) are documented, but the functions of N-glycans in development and disease are poorly understood (Hakomori, 1989). Studies on somatic tumor cell mutants have causally linked the GlcNAc-TV activity and its products, β1,6GlcNAc-branched N-glycans with increased metastatic potential (Yagel et al., 1989; Yousefi et al., 1991; Myc et al., 1989). The β1,6GlcNAc branch of N-glycans is the enzymatically preferred acceptor site for polylactosamine chain addition and enhanced expression of polylactosamine sequences on the cell surface is suggested to reduce cell adhesion to substratum and enhance organ colonization by tumor cells.

Lymphocyte adhesion may be similarly affected by β1,6GlcNAc-branched N-glycans, with consequences for immune cell maturation, activation and migration. Indeed, lymphoid cell functions are known to be sensitive to changes in glycosylation (Baum et al., 1995; Jiang et al., 1995; Lasky et al., 1989). A rare human congenital syndrome, LADII (Leukocyte adhesion deficiency II) caused by a defect in the synthesis of GDP-fucose, is clinically characterized by immunodeficiency and impaired wound healing (Phillips et al., 1995). Mice deficient in α1,3Fuc-TVII, which is required for the synthesis of Sle⁴ antigen in neutrophils and lymphocytes (Maly et al., 1996), have elevated peripheral leukocyte counts and are deficient in leukocyte extravasation into areas of inflammation (Maly et al., 1996). Gal:α2,6 sialyltransferase (ST6Gal) synthesizes SAα2,6Galβ1,4 sequences recognized by CD22 on the surface of B cells.
(Powell et al., 1993). Mice lacking this enzyme are severely immunosuppressed and display reduced serum IgM levels, impaired B cell proliferation and attenuated antibody production (Hennet et al., 1998). Transgenic mice expressing the O-glycan core 2 GlcNAc-T under the regulation of the lymphocyte-specific Lck promoter, show reduced T cell activation (Tsuboi and Fukuda, 1997).

To examine the function of GlcNAc-TV dependent glycosylation in murine biology, mice deficient in this enzyme were generated by targeted mutation of the Mgat5 locus. This chapter summarizes the phenotypic analysis of GlcNAc-TV deficient mice. Homozygous Mgat5−/− mice were viable, fertile and lacked β1,6GlcNAc-branched N-glycans. Although these mice showed no gross abnormalities, detailed histological examination revealed lymphocytic infiltrates in liver and kidney of Mgat5−/− mice, which were not present in wild type littermate and cage-mate animals.

GlcNAc-TV deficient mice displayed enhanced hypersensitivity to skin irritants, but the onset of the inflammatory response was slower. This was likely due to a reduced rate of leukocyte migration in vivo, which was associated with increased adhesion to substratum, measured in vitro. Sensitivity and cooperativity of T cell receptor (TCR)-dependent activation, as well as the rate of receptor internalization and actin reorganization, were increased in GlcNAc-TV deficient T cells. We also demonstrated that Mgat5 gene expression and β1,6GlcNAc-branched N-glycans increase with T cell activation. Therefore, levels of β1,6GlcNAc-branched N-glycans are regulated by the status of T cell activation. Consequently, our results suggest that β1,6GlcNAc-branched N-glycans have a direct role as a negative regulator of T cell response to antigen.
RESULTS

Generation of Mgat5\textsuperscript{-} mice: The Mgat5 targeting vector was designed to replace the coding portion of the first coding exon of Mgat5 with the lacZ reporter gene (Figure 1A,B). GlcNAc-TV enzyme activity was approximately 50% in heterozygous mice, and below the level of detection in Mgat5\textsuperscript{+} mice (Figure 1C,D). The products of GlcNAc-TV, β1.6GlcNAc-branched N-glycans, were also absent in the organs of Mgat5\textsuperscript{-} mice, as determined by L-PHA lectin blotting (Figure 1E). L-PHA binds to tri' and tetraantennary N-glycans, the synthesis of which is dependent on a substitution by GlcNAc-TV.

As expected, the LacZ expression pattern closely matched Mgat5 expression, which we have previously characterized by RNA in situ hybridization (Figure 2, Figure 3A)(Granovskyy et al., 1995). LacZ expression was observed in the giant cells of the trophoblast at E7.5 (Figure 2A). At E13.5, lacZ expression was observed in the crypts of the developing gut (Figure 2B), the medial and marginal zones of the developing brain (Figure 2C), the spinal chord and the skin (Figure 2D). LacZ staining was specific to neural cells in newborn and adult brain, and corresponded with L-PHA staining of neural axonal trees in Mgat5\textsuperscript{+/+} CNS (Figure 2E,F). In adult kidney, staining was observed in the podocytes of the glomerulli (Bowman's capsule), corresponding to the previously observed Mgat5 transcripts in this tissue. As expected, LacZ expression was significantly stronger in Mgat5\textsuperscript{-} embryos than in Mgat5\textsuperscript{++} embryos.

Mgat5\textsuperscript{-} mice were fertile and showed no overt abnormalities. However, histological and biochemical examination of tissues revealed several subtle abnormalities.
Figure 1: Generation and characterization of GlcNAc-TV deficient mice.

(A) Strategy for the inactivation of the *Mgat5* gene. The structure of the wild type *Mgat5* locus, the targeting vector, and the resulting targeted locus are illustrated. Nucleotides –22 to 241 of the coding portion of the first coding exon were replaced with lacZ and a neomycin-resistance gene; filled region represents the exon, 5' external probe is indicated by a solid bar above the *Mgat5* genomic locus. P, PstI restriction site. (B) Southern blot analysis of genomic DNA of F2 offspring derived from heterozygous crosses. PstI-digested DNA was hybridized with the 5' external probe. Lanes 2 and 4 show the *Mgat5* -/- genotype of F2 mice generated from two independently targeted ES clones. (C) GlcNAc-TV enzyme activity measured in *Mgat5* +/+ and *Mgat5* -/- tissue homogenates. Samples were assayed in duplicate and the assays were repeated on tissues from mice of both lines. (D) Time course of GlcNAc-TV enzyme activity in small intestine from *Mgat5* +/+ , *Mgat5* +/- and *Mgat5* -/- mice. Results represent duplicate assays. (E) L-PHA reactive glycoproteins in homogenates of I, intestine; K, kidney; H, heart; B, brain; S, spleen; Lu, lung and Li, liver separated by SDS-PAGE.
Figure 2: **LacZ expression from the mutant *Mgat5* allele is identical to the *Mgat5* expression pattern.**

LacZ expression pattern in murine embryos at E7.5 and at E13.5, and lacZ expression and L-PHA reactivity in adult cerebellum. LacZ expression was visualized by β-galactosidase cleavage of x-gal, which stains cells blue. (A) E7.5 embryo. The embryo proper does not express lacZ, but the giant cells of the trophoblast show strong expression. E13.5 *Mgat5* mutant embryos were stained for β-galactosidase activity (B-D). (B) E13.5 gut, staining in the crypts. (C) E13.5 mid-brain, where lacZ expression is seen in the medial and marginal zones. (D) E13.5 spinal cord and dermis express lacZ. (E) LacZ expression is observed in the Purkinje cells, a neural cell type in the adult cerebellum. (F) The lacZ expression in the Purkinje cells is matched by FITC-L-PHA reactivity of the dendritic trees of these cells, staining green. e, embryo; t, trophoblast; v, ventricle; m, medial and marginal zone of the 3rd ventricle; d, dermis; s, spinal chord; p, Purkinje cells; dp, dendritic trees of Purkinje cells.
Figure 3: Aberrant histology in \textit{Mgst5}^{+/} mouse tissues.

(A,B,C,) Photomicrographs of kidney from 9 months old mice. (A) LacZ staining of \textit{Mgst5}^{+/} kidney section, showing intense staining in podocytes, the filtering cells of the glomerulus. (B) \textit{Mgst5}^{+/} glomerulli. (C) \textit{Mgst5}^{+/} glomerulli. \textit{Mgst5}^{+/} glomerulli show a reduction of 47\% in nuclei when normalized to the area of the glomerulus. (D) Lymphocytic infiltrates, indicated by the arrow, in the liver of a 3 months old \textit{Mgst5}^{+/} mouse. (E) Lymphocytic infiltrates, indicated by the arrow, in the kidney of a \textit{Mgst5}^{+/} mouse.
Table 1: Summary of the phenotypic aberrations observed in \textit{Mgat5}^{-/-} mice.

<table>
<thead>
<tr>
<th>Affected system</th>
<th>Phenotype</th>
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<td><strong>Neurological</strong></td>
<td>15 / 18 \textit{Mgat5}^{-/-} females fail to nurse their young and are aggressive towards them (4 / 31 \textit{Mgat5}^{+/-} and 1 / 5 \textit{Mgat5}^{+/+} females exhibited similar behavior).</td>
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<tr>
<td><strong>Kidney</strong></td>
<td>Glomeruli have 28±7% fewer cells/area in 3 months old mice, and 47±15% fewer cells/area in 9 months old mice</td>
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<tr>
<td><strong>Lymphoid</strong></td>
<td>Lymphocytic infiltrates in solid organs.</td>
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The cellularity of kidney glomerulli was reduced by 28% in 3 months old \textit{Mgat5}^{-/-} animals, and by 47% in 9 months old \textit{Mgat5}^{+/+} animals, as compared to wild type littermates (Figure 3A-C). In addition, a strain dependent behavioral abnormality was observed in \textit{Mgat5}^{-/-} nursing females. 129/sv \textit{Mgat5}^{-/-} females (15 / 18) behaved in a negligent or aggressive manner towards their pups, failing to nurse and cannibalizing them. Only 4 / 31 \textit{Mgat5}^{+/+} and 1 / 5 \textit{Mgat5}^{++/} 129/sv females behaved similarly (results summarized in Table 1). Lack of nurturing behavior was not apparent on mixed 129/sv-CD1 or 129/sv-C57BL/6 strain background.

Lymphocytic foci were detected by histology in the liver and kidney at 3 months of age, but wasting or mortality due to autoimmune disease was not observed (Figure 3D,E). Peripheral white cell and erythrocyte counts were normal, and populations of T and B cells in spleen, thymus, and lymph nodes were also in the normal range by FACS analysis.

\textit{GlcNAc-TV deficiency causes hyperinflammatory response:} The lymphocyte infiltrates in \textit{Mgat5}^{-/-} mice suggest they may respond aberrantly to immune stimulants. To examine T cell dependent responses in vivo, a type IV (T cell dependent) delayed-type hypersensitivity (DTH) reaction was induced in mutant and \textit{Mgat5}^{++/} mice. The chemical antigen oxazolone was applied topically to the backs of the mice, then again 4 days later to the right ear (Correll et al., 1997). Ear swelling in \textit{Mgat5}^{++/} mice peaked 24 h after application of oxazolone to the ear, and returned to normal by day 5. Ear swelling in \textit{Mgat5}^{-/-} mice attained a higher maximum between 48 and 72 h, and persisted until day 5 (Figure 4A).
Figure 4: Inflammation in $\text{Mgat5}^{-/-}$ and littermate controls.

(A) DTH response in $\text{Mgat5}^{-/-}$ and $\text{Mgat5}^{+/+}$ mice exposed to oxazolone first on their back, then 4 days later on the right ear. The results are plotted as mean change ± S.E. in ear thickness relative to the vehicle-treated left ear for 7 $\text{Mgat5}^{-/-}$ mice and 6 $\text{Mgat5}^{+/+}$ littermate controls (★denotes p<0.001). (B) Ear swelling induced by topical application of arachidonic acid in $\text{Mgat5}^{-/-}$ and $\text{Mgat5}^{+/+}$ mice. The results are the average of five mice per genotype. One month later, when swelling had subsided completely, the experiment was repeated with the same mice, reversing the control and experimental ears on each mouse. The results were identical (★denotes p<0.001).
A single application of arachidonic acid, which is converted to leukotriene B4, a potent chemotactic agent for infiltrating neutrophils, macrophages and T lymphocytes, was used as a model of immediate skin hypersensitivity (Jiang et al., 1998). As shown in Figure 4B, ear swelling induced by arachidonic acid was also significantly greater and more persistent in \( Mgat5^{+/} \) mice. Maximum swelling was observed 2 h post application in the \( Mgat5^{+/} \) mice but was delayed until 7 h in the \( Mgat5^{-/-} \) mice.

**Cell migration is slowed and adhesion to fibronectin is enhanced in \( Mgat5^{-/-} \) mice:** The delayed onset of inflammation observed in \( Mgat5^{-/-} \) mice may be due to a glycosylation-dependent change in leukocyte adhesion. To examine this possibility, the adjuvant thioglycollate was injected i.p. and elicited peritoneal leukocytes were counted 3, 7 and 30 hrs later (Maly et al., 1996). Leukocyte infiltration was reduced at all time points in the \( Mgat5^{-/-} \) mice (Figure 5A). Indeed, at 30 hrs, the inflammatory response was dampened to basal levels in \( Mgat5^{-/-} \) mice but was still rising in \( Mgat5^{+/+} \) and \( Mgat5^{+/} \) mice. Fuc-TVII deficient mice lack selectin ligand and show a similar impairment in leukocyte extravasation (Maly et al., 1996). Leukocytes accumulate in blood of Fuc-TVII null mice due to impaired selectin-mediated attachment to endothelium, while leukocyte accumulation in \( Mgat5^{-/-} \) mice occurs in solid tissues and may be due to altered cell-substratum adhesion. Indeed, the thioglycollate-elicited leukocytes from \( Mgat5^{-/-} \) mice attached more avidly to fibronectin coated plastic, as did activated T cells (Figure 5B,C).

**GlcNAc-TV activity may regulate integrin signaling:** Leukocyte adhesion to fibronectin is mediated primarily by integrins VLA4 (\( \alpha 4\beta 1 \)) and VLA5 (\( \alpha 5\beta 1 \)), and the latter is known to be modified with \( \beta 1,6\)GlcNAc-branched glycans (Demetriou et al., 1995).
Figure 5: Cell migration is slowed and adhesion to fibronectin is enhanced in $Mgat5^-$ mice.

(A) Leukocyte motility into the peritoneal cavity of mice injected i.p. with thioglycollate. Cell counts represent the average ± ME of results for three mice per genotype. (B) Thioglycollate-activated leukocytes from $Mgat5^{+/+}$ and $Mgat5^-$ mice were seeded on various concentrations of fibronectin. The results represent the average ± SD of duplicate wells, and the experiment was repeated twice. (C) Adhesion of leukocytes and activated T cells to fibronectin-coated plastic. Neutrophils were activated by thioglycollate, and purified splenic T cells were activated with 40 ng/ml of PMA. Cells from 3 mice per genotype were assayed for adhesion, and the experiments were done twice.
Cell adhesion on fibronectin

**A**

- **Fibronectin (1 µg/well)**
- **Time (hr)**
- **Cells/ml (x10^4)**

**B**

- **Cells/grid**

**C**

- **T cells**
  - WT
  - Mlg5+/+
  - Mlg5+/-

- **Neutrophils**
  - WT
  - Mlg5+/+
  - Mlg5+/-

- $p > 0.01$

<table>
<thead>
<tr>
<th>Group</th>
<th>Cells/grid</th>
<th>Cells/ml (x10^4)</th>
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<tr>
<td>WT</td>
<td>73 ± 17</td>
<td>279 ± 45</td>
</tr>
<tr>
<td>Mlg5+/-</td>
<td>134 ± 31</td>
<td>153 ± 38</td>
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*Note: The table contains averages and standard deviations for different cell types and groups.*
Cell motility is dependent upon formation and turnover of integrin-mediated focal adhesions (Palecek et al., 1997). These structures can be readily examined in embryonic fibroblasts plated on fibronectin. *Mgat5*+/− fibroblasts spread more and pseudopodia showed fine actin microfilament, while *Mgat5*−/− cells had coarse actin stress fibers characteristic of non-motile cells (Figure 6A,B). The distribution of the cytosolic protein paxillin, which binds β1 integrin and the signaling molecules focal adhesion kinase (FAK), Csk and pp60Src at discrete sites of cell attachment in spreading and motile cells (Schaller et al., 1995), was also different in *Mgat5*+/− and *Mgat5*−/− cells. In *Mgat5*+/− cells, paxillin localized to the distal ends of microfilaments characteristic of focal adhesions, but the protein was dispersed in the cytoplasm of *Mgat5*−/− fibroblasts. Thus, GlcNAc-TV-dependent glycosylation may enhance focal adhesion formation in vitro, and may account for the observed motility of *Mgat5*+/− leukocytes in vivo.

*Mgat5*−/− T-splenocytes are hypersensitive to T cell mitogens: The exaggerated and persistent DTH response in *Mgat5*−/− mice was reflected in the behavior of splenocytes in vitro. Spleen cells were stimulated with mitogens applied in solution. Mitogens used were the lectins leukoagglutinin (L-PHA) and Concanavalin A (ConA), and soluble monoclonal antibodies reactive to CD3, clone 2C11 (anti-CD3), and to TCRα/β, clone H59.72 (anti-TCR). Following stimulation with ConA, anti-CD3 or anti-TCR, T cell proliferation was 3-5 fold greater for spleen cells from *Mgat5*−/− compared to *Mgat5*+/+ littermates (Figure 7A-C). In contrast, *Mgat5*−/− splenocytes were completely unresponsive to L-PHA (Figure 7D). Thus, these results demonstrate that T cell activation by L-PHA requires the presence of surface β1,6GlcNAc-branched N-glycans.
Figure 6: Focal adhesions in embryonic fibroblasts plated on fibronectin.

*Mgat5*+/- (A,C) and *Mgat5*^-/- (B,D) fibroblasts were stained with rhodamine-phalloidin, FITC conjugated anti-paxillin antibodies, and Hoechst 33258 stain. Fluorescence images of the cells were obtained using a deconvolution microscope and digital data capture. Paxillin (green) is localized to ends of microfilaments (red) in focal adhesions of *Mgat5*+/-, but not in *Mgat5*^-/- fibroblasts. *Mgat5*^-/- fibroblasts spread to cover a greater surface area than *Mgat5*+/- cells, and the nucleus in panel A is out of view to the left. Panels A and B are at 100X magnification, and panels C and D are at 40X magnification.
**Figure 7:** *Mgat5−/−* T splenocytes are hypersensitive to T cell mitogens.

*Mgat5−/−* (●) and *Mgat5+/* (□) spleen cells were cultured at 2 x 10⁵ cells/ml and stimulated with increasing concentrations of (A) anti-TCRαβ (B) anti-CD3 (C) Con A (D) L-PHA (E) PMA (10 ng/ml) plus ionomycin (0.5μg/ml) and (F) LPS. Data in all panels represent the average ± SD of triplicate measurements and experiments were repeated 3 times. The experiments shown in panel C and D were repeated with mice generated from the second targeted ES cell line, and with the *Mgat5* mutation on the mixed 129/sv-CD1 and 129/sv-C57black6 background with identical results.
Figure A: Graph showing the effect of varying concentrations of anti-TCR antibody (α-TCR Ab) on the incorporation of \(^{3}H\)-thymidine (cpm). The x-axis represents the concentration of α-TCR Ab (μg/ml), and the y-axis represents the incorporation of \(^{3}H\)-thymidine (cpm).

Figure B: Graph showing the effect of varying concentrations of anti-CD3 antibody (α-CD3 Ab) on the incorporation of \(^{3}H\)-thymidine (cpm). The x-axis represents the concentration of α-CD3 Ab (μg/ml), and the y-axis represents the incorporation of \(^{3}H\)-thymidine (cpm).

Figure C: Graph showing the effect of varying concentrations of Con A on the incorporation of \(^{3}H\)-thymidine (cpm). The x-axis represents the concentration of Con A (μg/ml), and the y-axis represents the incorporation of \(^{3}H\)-thymidine (cpm).

Figure D: Graph showing the effect of varying concentrations of L-PHA on the incorporation of \(^{3}H\)-thymidine (cpm). The x-axis represents the concentration of L-PHA (μg/ml), and the y-axis represents the incorporation of \(^{3}H\)-thymidine (cpm).

Figure E: Bar graph comparing the incorporation of \(^{3}H\)-thymidine (% of control) between Mgp5+/+ and Mgp5/-. The black bar represents Mgp5+/+ and the white bar represents Mgp5/-. The x-axis represents the concentration of LPS (μg), and the y-axis represents the incorporation of \(^{3}H\)-thymidine (% of control).
Mgat5<sup>+/+</sup> and Mgat5<sup>−/−</sup> spleen cultures treated with phorbol 12-myristate 13-acetate (PMA) and the Ca<sup>2+</sup> ionophore, ionomycin, showed similar levels of proliferation indicating that intracellular signaling downstream of these mediators is normal in Mgat5<sup>−/−</sup> cells (Figure 7E). The Mgat5<sup>−/−</sup> phenotype was cell-type specific, as B cell responses to lipopolysaccharide LPS (Figure 7F) and to anti-IgM or anti-CD40 antibodies, in the presence or absence of interleukin-4 (data not shown), were similar in mutant and wild type littermates.

GlcNAc-TV deficiency is synergistic with CD28 co-receptor stimulation: CD28 co-receptors on T cells stabilize TCR-mediated signaling aggregates and reduce the number of engaged TCR required to propagate intracellular signals and initiate cell proliferation (Bachmann et al., 1997; Wulfing and Davis, 1998a; Wulfing and Davis, 1998b). Mgat5 dependent glycosylation of T cell surface glycoproteins may also affect the kinetics of agonist-induced TCR aggregation and signaling. To examine this possibility, purified T cells were cultured at low density and stimulated with increasing concentrations of soluble anti-CD3 in the presence or absence of anti-CD28 antibodies (anti-CD28). Both the GlcNAc-TV deficiency and anti-CD28 reduced the requirements for TCR agonist as indicated by D<sub>50</sub> values (Figure 8A,B). Furthermore, the apparent Hill coefficient (n<sub>H</sub>), a measure of cooperativity, was increased both by the GlcNAc-TV deficiency and by the addition of anti-CD28. The Hill coefficient for the TCR dependent activation of wild-type T cells in the absence of co-receptor stimulation was 6. GlcNAc-TV deficiency increased the apparent cooperativity by 5.5 fold, and this effect was synergistic with that of CD28 co-receptor engagement (Figure 8B). Cooperativity or ultrasensitivity in signaling pathways adds precision to "switch-like" events (measured here as entry into S phase).
Figure 8: GlcNAc-TV deficiency Enhances Cooperativity of TCR-mediated cellular activation.

(A) T cell activation by anti-CD3 antibodies in the presence or absence of anti-CD28 antibodies (0.5 μg/ml) for Mgat5−/− and Mgat5+/+ cells. Cultures were seeded at 5 x 10^4 cells/ml. (B) Summary of the kinetics of responses in panel A. Hill coefficients were calculated using log(y/1-y) = log Ka + n log A, where Ka is the Hill coefficient and A is substrate concentration.
A

$^{3}H$-thymidine (cpm)

\[ \alpha-CD3 \ (\mu g/ml) \]

B

**D50 and $n_H$ values for TCR response**

<table>
<thead>
<tr>
<th>genotype</th>
<th>$\alpha$CD28</th>
<th>$D_{50}$ (ng/ml)</th>
<th>$n_H$</th>
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<tbody>
<tr>
<td>+/-</td>
<td>+</td>
<td>4±0.5</td>
<td>59.6</td>
</tr>
<tr>
<td>+/-</td>
<td>-</td>
<td>11±1.8</td>
<td>33.8</td>
</tr>
<tr>
<td>++/+</td>
<td>+</td>
<td>12±0.8</td>
<td>16.7</td>
</tr>
<tr>
<td>++/+</td>
<td>-</td>
<td>~36</td>
<td>~6.0</td>
</tr>
</tbody>
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A similar mechanism was recently described for progesterone triggering of meiosis in Xenopus oocytes (Ferrell and Machleder, 1998). Thus, GlcNAc-TV-dependent glycosylation has a significant influence on agonist threshold and cooperativity of TCR signaling.

**GlcNAc-TV deficiency directly affects antigen-induced TCR internalization:** We reasoned that formation of TCR receptor aggregates in the plane of the plasma membrane is a source of ultrasensitivity that may be regulated by glycosylation. A minimal or threshold number of TCR must be engaged to initiate entry into S phase (Bachmann et al., 1997; Wulfing and Davis, 1998a; Wulfing and Davis, 1998b), followed by rapid cytoskeletal reorganization and TCR internalization (Fischer et al., 1998; Viola and Lanzavecchia, 1996). To determine whether the *Mgat5*<sup>-/-</sup> mutation affects TCR movement in response to antigen, we measured TCR internalization induced by anti-CD3 or by PMA plus ionomycin. Cell surface TCR levels in unstimulated *Mgat5*<sup>+/+</sup> and *Mgat5*<sup>-/-</sup> T cell populations were comparable. TCR internalization induced by anti-CD3 proceeded more rapidly in GlcNAc-TV deficient cells compared to *Mgat5*<sup>+/+</sup> cells with a half maximal internalization observed at 1.5 and 2.2 min, respectively (Figure 9A). Maximum TCR internalization was approximately 2.4 fold greater in *Mgat5*<sup>-/-</sup> cells compared to *Mgat5*<sup>+/+</sup> T cells. Consistently, TCR ligation induced a more rapid depolymerization of actin filaments, an event that precedes the first step in actin polymer reorganization, in *Mgat5*<sup>-/-</sup> T cells compared to wild type cells. MAPK phosphorylation, a downstream effector of TCR signaling, also peaked more quickly and reached higher levels in GlcNAc-TV deficient T cells (Figure 9B). Phosphorylated PKB, a kinase downstream of both TCR and integrin signaling, was abnormally low in resting *Mgat5*<sup>-/-</sup> T cells, but increased after stimulation to levels observed in *Mgat5*<sup>+/+</sup> cells (Figure 9B).
In addition, cytoskeletal reorganization, as visualized by the breakdown of actin polymers, was faster in Mgat5<sup>-/-</sup> T cells than in wild type cells (Figure 9C).

PMA stimulation results in CD3 recycling to the cell surface, and endocytic degradation of CD4 (Ruegg et al., 1992). To examine endocytosis in Mgat5<sup>-/-</sup> cells, we monitored CD3 and CD4 levels in PMA-activated T cells. Although PMA induced optimal cell proliferation, CD3 internalization was only weakly induced, and significantly, was similar for both Mgat5<sup>+/+</sup> and Mgat5<sup>-/-</sup> T cells (Figure 9D). In contrast, 80% of CD4 was internalized and degraded in lysosomes compared to unstimulated cell-surface levels in all three genotypes (Ruegg et al., 1992). Thus, endocytosis in Mgat5<sup>-/-</sup> T cells does not differ from endocytosis in Mgat5<sup>+/+</sup> T cells. Rather, Mgat5<sup>+/+</sup> and Mgat5<sup>-/-</sup> T cells differ at the level of extracellular TCR ligation, the primary force directing receptor aggregation and internalization.

**Mgat5 expression is regulated by T cell activation:** GlcNAc-TV enzyme activity increases with activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, possibly through the activation of Mgat5 transcription by the Ras/MAPK/Ets pathway (Buckhaults et al., 1997; Lemaire et al., 1994; Lu and Chaney, 1993). Consistent with this possibility, β-galactosidase activity produced by the gene product of the inserted lacZ reporter gene in Mgat5<sup>-/-</sup> T cells increased 5 fold following T cell activation for 48 hours. Furthermore, this resulted in altered glycosylation of the cell surface, as L-PHA reactivity also increased in Mgat5<sup>+/+</sup> T cells following activation (Figure 10A,B).
Figure 9: GlcNAc-TV deficiency directly affects antigen-induced TCR internalization.

(A) T cells from $Mgat^5^{-/}$ or $Mgat^5^{+/+}$ mice were stimulated with 0.1 μg/ml anti-CD3, and cell surface levels of TCR (relative mean flourescent intensity, MFI) were measured by FACS analysis using FITC-anti-TCR$_{αβ}$. The same result was obtained when the roles of anti-TCR$_{αβ}$ and anti-CD3 were reversed. (B) Phosphorylated MAPK and Akt/PKB in T cells following addition of anti-CD3 antibodies. The numbers represent the ratio of phosphorylated MAPK and PKB normalized to time 0 for each series. (C) Breakdown and reorganization of actin was measured by rhodamine-phalloidin binding to actin bundles following the addition of 0.1 μg/ml anti-CD3. The results represent duplicate measurements. (D) Splenocytes from $Mgat^5^{-/}$ mice and from $Mgat^5^{+/+}$ controls, stimulated with 10 ng/ml PMA, were stained with FITC-anti-CD3 and PE-anti-CD4. No significant difference in staining with either antibody was observed between the genotypes.
Figure 10: *Mgat5* expression is regulated by T cell activation.

(A) L-PHA binding to *Mgat5*+/+ T lymphocytes either untreated (white) or stimulated with 0.1 μg/ml of anti-CD3 and 0.5 μg/ml anti-CD28 for 48h (grey). (B) LacZ activity in untreated (white) and anti-CD3 and anti-CD28 stimulated (grey) T cells from *Mgat5*−/− mice.
**DISCUSSION**

*GlcNAc-TV deficient mice:* We generated mice deficient in GlcNAc-TV activity to study the biological role of β1,6GlcNAc-branched N-glycans. *Mgat5* message, GlcNAc-TV activity and β1,6GlcNAc-branched N-glycans were not detected in mice homozygous for the *Mgat5* targeted mutant allele. Therefore, these mice are functionally null indicating that only one gene encodes GlcNAc-TV activity.

GlcNAc-TV deficient mice are viable and fertile, unlike GlcNAc-TI deficient mice, which do not survive past E9.5 (Ioffe and Stanley, 1994; Metzler et al., 1994). Ioffe et al. have demonstrated that maternal message of *Mgat1* is present in post-implantation embryos and, therefore, possibly rescues the embryos from an even earlier death (Ioffe et al., 1997). Although *Mgat5* is highly expressed in the embryo from the age of E9.5 (Granovsky et al., 1995), lack of GlcNAc-TV activity does not result in significant developmental retardation or abnormalities. GlcNAc-TV deficient females produce normal-sized litters when mated with males of all *Mgat5* genotypes, suggesting that, indeed, *Mgat5* message is not necessary for normal development or fertility. At E7.5, *Mgat5* transcripts are expressed in the giant cells of the trophoblast, an extra-embryonic invasive cell type, phenotypically similar to transformed cells (Granovsky et al., 1995; Hoffman and Wooding, 1993). Since the O-glycan core 2 GlcNAc-T is also expressed in these cells at the same developmental stage (Granovsky et al., 1995), it is possible that β1,6GlcNAc-branched O-glycans are able to functionally compensate for the lack of β1,6GlcNAc-branched N-glycans. Even though N-glycans and O-glycans are present on different glycoproteins and fulfill distinct biological roles, the common extension of the β1,6GlcNAc-branch by polylactosamine chains may be the functionally important structural feature of glycans in the giant cells of the trophoblast, enabling O-
glycans to rescue the $Mgat5^{-/}$ cells. A cross between core2 GlcNAc-T deficient mice and $Mgat5^{-/}$ mice may address this question.

Although GlcNAc-TV deficient 129/sv female mice produced normal litters, the majority of these mice failed to nurse their young. This deficiency could have been due to either a lactation defect or to a behavioral abnormality. Mammary development appeared normal in $Mgat5^{-/}$ females (see example in Figure 3, chapter 4), and milk was evident in the mammary glands of several $Mgat5^{-/}$ females that were examined within 24 hours of giving birth. These data, and the observed aggressiveness towards their pups, suggest that the failure of $Mgat5^{-/}$ 129/sv females to nurse their young is due to a neurological defect. Nurturing behavior defects have been ascribed to neural abnormalities in previously characterized mutant mice. Mice deficient in FosB exhibit a nurturing defect, while showing no other cognitive deficiency (Brown et al., 1996). The aberrant behavior is likely due to the absence of FosB in the preoptic area, a region of the hypothalamus that is critical for nurturing. A more severe cognitive phenotype, which includes failure to nurture, is observed in Fkh-5-deficient mice (Wehr et al., 1997). Fkh-5 is a winged helix transcription factor and in adult mice it is expressed in the mammillary body region of the caudal hypothalamus and in the midbrain. $Mgat5$ is highly expressed in neural epithelium from E 9.5 and into adulthood (Granovsky et al., 1995) and expression is detected in the hypothalamus. In the adult mouse brain, its expression is restricted to neural cells and is absent from the supporting glial cells (Figure 2E). However, histological examination of $Mgat5^{-/}$ central nervous system reveals no obvious anatomical abnormalities, suggesting that the defect in behavior may be a biochemical defect in some aspects of potentiation (personal communication, J. Henderson).
Mgat5 deficiency affects several cell types and tissues that have been previously identified as high in GlcNAc-TV activity (Perng et al., 1994). In addition to the behavioral defect, the Mgat5⁻/⁻ mutation affects the kidney and the lymphoid system. These findings suggest that GlcNAc-TV activity might be necessary for cellular processes common to many cell types.

The lymphocytic infiltrates, observed in the histological examination of Mgat5⁻/⁻ mouse tissues, prompted us to investigate the inflammatory responses of these mice. Arachidonic acid is the metabolic precursor molecule for the production of prostaglandins and leukotrienes, which are vasodilators and leukocyte-attracting chemokines, respectively. Topical application of arachidonic acid induces a rapid migration of leukocytes to the site of application, and the subsequent attraction and activation of T cells several hours later. Inflammation in response to arachidonic acid was delayed but stronger in Mgat5⁻/⁻ mice compared to wild type littermates. These results suggest that the initial rate of leukocyte migration to the site of application may be slowed, but that once leukocytes and lymphocytes are attracted, they are stimulated to a greater degree and their stimulation lasts longer. The specific hyperreactivity of T cells was confirmed by the increased response in Mgat5⁻/⁻ mice to oxazolone, which induces a T cell-mediated DTH reaction (type IV). Therefore, the results of these two hypersensitivity models suggest that leukocyte migration and T cell activation may be aberrant in Mgat5⁻/⁻ mice. Interestingly, a full inflammatory response was not mounted when Mgat5⁻/⁻ mice were injected with thioglycollate. Indeed, the inflammatory response appeared to be over at 30 hrs, while the inflammatory response in wild type mice was still rising. Thioglycollate stimulation results in the inhibition of arachidonic acid release, with subsequent reduction in the production of arachidonic acid metabolites, but with enhanced production of TNFα (Carrick et al., 1995). Thus, it is possible that the Mgat5⁻/⁻
mice may be hypersensitive to arachidonic acid metabolites, but hyposensitive to thioglycollate and TNFα.

**GlcNAc-TV deficiency alters cellular adhesion and migration:** In this study, we observed that fewer leukocytes migrated into the peritoneum when induced with thioglycollate. Both peritoneal leukocytes and PMA-activated T cells were more adherent to fibronectin in vitro. These results suggest that the observed delay in the inflammatory response to arachidonic acid may be due to slower leukocyte migration resulting from stronger cellular adhesion to fibronectin.

GlcNAc-TV deficient fibroblasts exhibited intracellular architecture consistent with stationary cells, including heavy actin stress fibers, lack of localization of focal adhesion proteins and reduced spreading. *Mgat5−/−* fibroblasts in serum-free conditions, as well as freshly harvested resting T cells, had aberrantly low levels of phosphorylated PKB. PKB is a kinase downstream of PI3K, the activation of which is important for integrin-mediated cytoskeletal reorganization that allows focal adhesion turnover, membrane ruffling and subsequent migration (Clark and Brugge, 1995; Parent and Devreotes, 1999; Stephens et al., 1998). The α5β1 integrin heterodimer (VLA-5) is the primary cellular receptor for fibronectin, and both chains are subject to GlcNAc-TV-dependent glycosylation (Demetriou et al., 1995). Overexpression of GlcNAc-TV in MvLu1 cells resulted in enhanced cellular migration and decreased adhesion to ECM proteins *in vitro* (Demetriou et al., 1995). Therefore, the enhanced adhesion of GlcNAc-TV deficient leukocytes and T cells to fibronectin is consistent with the hypothesis that GlcNAc-TV activity may modulate VLA5 binding to fibronectin and its subsequent signaling.

**TCR signaling and β1,6GlcNAc-branched N-glycans:** We have shown that T cells from GlcNAc-TV deficient mice are hypersensitive to T cell mitogens. In contrast, B cells
from GlcNAc-TV deficient and wild type mice were stimulated similarly by a variety of B cell mitogens, indicating that the hypersensitivity is T cell specific. Therefore, β1,6GlcNAc-branched N-glycans may be present on and affect the biological activity of glycoproteins required exclusively for T cell function.

The majority of the cell surface molecules comprising the TCR/CD3 complex (TCR), and the co-receptor molecules CD4 and CD8, are glycosylated (Huppa and Ploegh, 1997) (for detailed description of TCR-mediated signaling see Figure 11)(Harris et al., 1990; Wu et al., 1996). CD45, a phosphatase important in the initiation of TCR signaling has L-PHA reactive structures, indicating it is subject to GlcNAc-TV mediated glycosylation. In addition, many of the reported costimulatory molecules, such as CD28 and LFA-1 (lymphocyte function-associated antigen-1) are also N-glycosylated (Bajorath et al., 1997; Miller and Springer, 1999; Monostori et al., 1997), although detailed structural analysis of the glycans is yet to be done. The crystal structure of the αβ chains of the TCR shows that 4 N-glycosylation sites are occupied on the β chain, and 3 are occupied on the α chain(Wang et al., 1998). Of these seven chains, 5 are present on the invariant regions (Ca and Cβ) (Wang et al., 1998). The carbohydrates of the α chain have been previously characterized as complex-type, a likely substrate for GlcNAc-TV (Hubbard et al., 1986).
The TCR is an oligomeric complex containing both antigen recognition subunits and signal transduction subunits. The αβ subunits of the T cell receptor recognize peptide in the context of a major histocompatibility complex (MHC) on the antigen presenting cell (APC), but they do not possess intracellular signaling domains (Huppa and Ploegh, 1997). The αβ chains are associated by salt bridges with CD3γ, CD3δ and CD3ε, known as the CD3 complex, and with a fourth protein, CD3ζ, essential for signaling (Janeway, 1992). The cytoplasmic domains of all four CD3 proteins contain sequences called immunoreceptor tyrosine-based activation sequence motifs (ITAMs) (Janeway, 1992). In addition to the stable TCR complex, a co-receptor molecule (CD4 or CD8) transiently associates with the complex during antigen recognition and enhances the sensitivity of T cells to antigen.

Antigen induced signaling through the TCR is initiated by bringing together the TCR, CD4 or CD8, and CD45, each of which is N-glycosylated (Zenner et al., 1995). CD45 is a tyrosine phosphatase which removes inhibitory phosphate groups and thereby activates the Lck and Fyn tyrosine kinases associated with the co-receptor and the TCR, respectively (Figure 10) (Clements et al., 1999). Lck phosphorylates ITAMs on CD3ζ, and the phosphorylated CD3ζ is then able to recruit ZAP-70 (zeta chain associated protein-70) (Clements et al., 1999). The activation of ZAP-70 triggers intracellular signaling cascades including the Ras pathway (Clements et al., 1999), and signaling through Ras culminates in the activation of Fos and the AP-1 transcription factors, initiating new gene transcription necessary for T cell differentiation, activation and proliferation.

TCR signaling is enhanced by the engagement of costimulatory molecules on the T cell by their ligands on the APC. In particular, the costimulatory effects of CD28 when engaged by the B7 ligands and of LFA-1 bound to I-CAM1 are well documented (Bachmann et al., 1997; Lenschow et al., 1996; Shahinian et al., 1993; Yashiro et al., 1999).
TCR chains
CD4/CD8 co-receptor
CD45 phosphatase
Fyn kinase
Lck kinase
ZAP 70
Phosphate
γ/δ/ε CD3 chains
Phosphate
CD28

\[ \text{α/β TCR chains} \]
\[ \text{γ/δ/ε CD3 chains} \]
\[ \text{ζ CD3 chain} \]

\[ \text{PI3 kinase} \]
\[ \text{CD28} \]

Antigen-MHC on APC
B7 ligand on APC

\[ \text{Ras signaling cascade} \]

- Cytoskeletal reorganization
- IL-2 production

\[ \text{Ca}^{2+} \]
\[ \text{PKC} \]

Phospholipase C
X-ray crystallography studies indicate that the N-glycan chains are not likely to obstruct peptide presentation to the TCR, but by projecting outward, away from the protein, they have the potential to influence TCR aggregation (Garcia et al., 1996; Wang et al., 1998).

Antigen induced signaling through the TCR is initiated by bringing together or clustering the TCR, CD4 or CD8, and CD45, each of which is N-glycosylated (Zenner et al., 1995). CD45 is a tyrosine phosphatase which removes inhibitory phosphate groups and thereby activates the Lck and Fyn tyrosine kinases associated with the co-receptor and the TCR, respectively (Clements et al., 1999). Clustering of receptor and co-receptor glycoproteins brings together the various phosphatases and kinases and their substrates and may be influenced by glycosylation. The bulk and charge of the glycan chains provides steric hindrance to protein-protein interactions, impeding receptor clustering. Alternatively, the mobility of the various molecules in the plane of the membrane might be influenced by the kinetics of sugar-lectin interactions in cis or in trans (i.e. single cell or between cells, respectively). Indeed, the addition of exogenous galectin 1 to cultured T cells antagonizes TCR signaling (Vespa et al., 1999). On B cells, the binding in cis of α2,6SA on polylactosamine to CD22 is essential for proper B cell maturation and IgM production (Hennet et al., 1998).

There are three possible mechanisms by which glycan-glycan or glycan-lectin interactions could modulate the physical interactions required for T cell activation. The formation of the initial TCR-CD4/CD8-CD45 complex, or subsequent TCR clustering could be impeded by glycosylation, thereby slowing the rate with which activated TCR aggregates are formed. Alternatively, carbohydrate chains may destabilize the activated complex, thereby reducing the time available for signaling, and, in effect, acting to counterbalance CD4 and CD8. A third possibility is that glycosylation alters the rate of
migration of the activated complex into kinase-rich "rafts", which are cholesterol-rich membrane microdomains where many signaling molecules important for TCR activation, such as ZAP-70, Lck and LAT are sequestered (Xavier et al., 1998). Slowed migration into the "rafts" lengthens the period between TCR engagement and its interaction with its signaling partners, thus reducing the probability of initiating T cell activation. The mechanisms mentioned above could apply equally well to the binding and intramembrane movements of N-glycosylated costimulatory molecules, leading to the modulation of their biological activity. A combination of these mechanisms may operate in a complex manner to produce the observed phenotypes.

TCR internalization in response to mitogen stimulation is increased in GlcNAc-TV deficient T cells (Figure 9A). TCR internalization has been previously used as a measure of TCR aggregation, a necessary step for TCR mediated signaling. TCR aggregation and internalization require cytoskeletal rearrangement, which occurs faster in M<sup>gat5</sup>- T cells compared to wild type T cells (Figure 9C). Cytoskeletal reorganization in T cells, which allows glycoprotein receptors to move in the plane of the membrane, plays a key role in TCR signaling (Lanzavecchia et al., 1999; Xavier et al., 1998). Specifically, antigen presentation by APCs results in the formation of ordered protein-rich domains at the interface between the APC and the T-cell, where TCR complexes and other signaling molecules, such as LFA-1 aggregate in distinct spatial distributions (Monks et al., 1998). In addition, efficient TCR signaling is dependent on the formation of kinase-rich "rafts" into which triggered TCRs are recruited (Xavier et al., 1998).

TCR agonists, such as anti-CD3 antibody and peptide-MHC on APCs induce TCR clustering, and the threshold of T cell activation, as measured by calcium influx, is 3 TCRs per cluster, or 1 TCR cross-linked to a co-receptor molecule (Boniface et al., 1999; Delon et al., 1999). When polymers of haptens or polypeptide oligomers of peptide
antigens were used to stimulate T cell activation, an increased potency was observed with increased number of antigenic units, reaching a plateau at about 16 units (Boniface et al., 1999). Thus, T cell activation is dependent on the cooperativity generated by receptor clustering, and our results indicate that glycosylation may regulate TCR clustering.

Cooperativity of T cell activation is also enhanced by the engagement of costimulatory molecules, such as CD28. CD28 engagement by B7 ligands results in the phosphorylation of CD28, and the activation of the tyrosine kinases Lck and EMT, thus acting to amplify TCR signaling by enhancing early phosphorylation events (Gibson et al., 1998). CD28 stimulation results in cytoskeletal rearrangement and raft aggregation (Viola et al., 1999), which appears to physically aid in sustaining TCR signaling. CD28 is heavily N-glycosylated, and lack of β1,6GlcNAc-branching may enhance this receptor’s engagement by the B7 ligands. Indeed, the effects of GlcNAc-TV deficiency and CD28 engagement on T cell activation were synergistic, suggesting that signaling mediated by both TCR and CD28 may be regulated by glycosylation.

The binding kinetics of effective APC-presented antigens are optimal when on-rates allow recruitment of TCR clustering, but dissociation occurs at a rate optimal for serial binding and recruitment of additional TCR molecules into the clusters. In this fashion, a few agonistic peptide-MHC complexes are able, with time, to engage and trigger a much larger number of TCRs by serially binding, triggering and disengaging from TCRs (Valitutti et al., 1995). Serial triggering is an important regulatory element in T cell activation, since a threshold number of triggered TCR must be achieved for activation. The threshold number is not absolute, and is subject to modification by costimulatory signals. For example, in order to activate human T-cell clones (~30,000 TCR/cell), 8,000 triggered TCRs were required in the absence of CD28 costimulation, but only 1,000 triggered TCRs were needed in its presence (Viola and Lanzavecchia,
1996). The engagement of TCR by peptide-MHC does not result in the immediate triggering of TCR signaling. Rather, TCR signaling requires a series of phosphorylation steps, leading to the recruitment and activation of ZAP-70, which activates TCR-mediated signaling (See Figure 11). The multiple phosphorylation steps act as a proofreading mechanism, since weakly bound peptides with fast off-rates do not remain bound to the TCR long enough to complete the multiple phosphorylation events, and therefore fail to trigger T cell activation. In this context, CD4 and CD8 coreceptor molecules may act to enhance activation by binding the MHC and stabilizing TCR-peptide-MHC interactions to allow weak agonists to engage TCRs long enough (~10 seconds) to reach the triggering threshold. Our study suggests that β1,6GlcNAc-branched N-glycans may destabilize the TCR complex, thus exerting the opposite effect to CD4 and CD8 engagement.

GlcNAc-TV deficient T-splenocytes of Mgat5−/− mice are quiescent and do not spontaneously proliferate in tissue culture in the absence of T cell mitogens. Thus, β1,6GlcNAc-branched N-glycans do not appear to be essential for maintaining quiescence, but are important in regulating T cell activation through altering the kinetics of TCR aggregation in response to agonist. In the absence of cell surface β1,6GlcNAc-branched N-glycans, TCR oligomerize more efficiently, thereby allowing activation to occur at lower agonist levels and with greater cooperativity. In contrast, PMA-induced activation, which bypasses the need for cell surface receptor ligation (Ruegg et al., 1992), is similar in mutant and wild type cells, suggesting that downstream signaling in Mgat5−/− T cells is intact. Therefore, the β1,6GlcNAc-branched N-glycans on the surface of T cells appear to attenuate receptor aggregation, and thereby may prevent or reduce spurious activation in the absence of high-affinity ligands in vivo.
Our results indicate that β1,6GlcNAc-branched N-glycans suppress T cell activation and that *Mgat5* gene expression is induced by T-cell activation. Therefore, GlcNAc-TV upregulation may provide negative feedback, suppressing TCR responses in activated T cells. Negative feedback may be required to suppress hyperactivation of T cells as they migrate into sites of infection where antigen concentration is higher. Hyperactivation would be detrimental, as it may cause premature apoptosis, leading to an unsustained and therefore ineffective T cell response. In addition, negative feedback is necessary for shutting down immune responses and inflammation once infection is cleared, and our results suggest that regulated expression of *Mgat5* in T cells is a potent means used to regulate this process.

**Immune regulation by *Mgat5***: The results presented in this chapter may have implications for hypersensitivities, allergies and the ability to clear infections. GlcNAc-TV deficiency resulted in slower onset of inflammation in the two models of hypersensitivities tested. Therefore, innate immunity may be compromised in these mice by the inability of leukocytes to migrate to the site of infection as rapidly as in wild type mice.

The profile of the immediate-type hypersensitivity response to arachidonic acid in *Mgat5*−/− mice was markedly different than the response in PPARα−/− mice, where the oxidative degradation of leukotriene B4 is disrupted (Devchand et al., 1996). The PPARα−/− mice did not differ from the wild type mice in the amplitude of the inflammatory response nor in the time during which maximal response was reached. However, due to the slower clearance of leukotriene B4 in the PPARα−/− mice, inflammation persisted longer. In the *Mgat5*−/− mice, the magnitude, the timing and the duration of the response were all significantly different from the response in wild type mice, suggesting that several processes may be perturbed by GlcNAc-TV deficiency.
The phenotype of the \( M_{gat5}^{+/} \) mouse is suggestive of the \( CD43^{+/-} \) mouse, both by the hypersensitivity of T cells and by the enhanced adhesion of T lymphocytes to fibronectin (Manjunath et al., 1995). In the \( CD43^{+/-} \) mouse, vaccinia virus was cleared less efficiently than in wild type mice, and the authors speculate that slowed migration, tissue localization or the post-lysis viral clearance by phagocytes may be impaired (Manjunath et al., 1995).

The hyperactivation of T cells in \( M_{gat5}^{+/} \) mice suggests that these mice may be susceptible to contact hypersensitivities and allergies. T cell mediated responses were not only higher in these mice, but were also slower to subside, suggesting that GlcNAc-TV activity is required for the efficient shutting down of an immune response. This defect may have implications for induced autoimmunity.

There is no data available on the variances in basal levels of GlcNAc-TV activity in the human population. There is also no information about the differences in maximal induction of the \( M_{gat5} \) gene expression following T cell activation. The results presented above suggest that both factors may contribute to the observed variability in human responses to immune challenges. In addition to changing T cell sensitivity to antigens, GlcNAc-TV activity changes the rate of cell migration, and therefore could potentially have implications for the ability to clear infections. Our results suggest that patients suffering from hypersensitivities and allergies would be of particular interest for studying their GlcNAc-TV activity profiles.
MATERIALS and METHODS

Mutation of the Mgat5 gene: A genomic library from strain 129/sv mice was screened with a Mgat5 cDNA probe. A 13.5 kb genomic clone containing the 205 nt 5' untranslated region and 241 nt spanning the first coding exon was used to construct the Mgat5 targeting vector. The Mgat5 targeting vector was constructed with LacZ replacing the coding region of the first exon. The targeting vector was linearized with NotI, electroporated into R1 ES cells, and transfected cells were selected in the presence of G418 and gancyclovir as previously described. DNA from drug resistant colonies was digested with PstI and screened for homologous recombination by Southern blot analysis using a 1.7 kb PstI/XbaI genomic fragment external to the targeting vector. Two Mgat5+/− ES cell lines were aggregated with blastocysts from CD-1 mice, and implanted into pseudo-pregnant CD-1 females. The resultant chimeras were mated with 129/sv females. Heterozygous progeny were intercrossed to generate Mgat5−/− mice, and experiments were performed on the 129/sv background. For histological examination, mice were perfused with 10% buffered formalin in vivo, tissues were paraffin embedded, sectioned and stained with hematoxylin and eosin. GlcNAc-TV enzyme activity in tissue homogenates was determined as transfer of [3H]-GlcNAc from UDP-[3H]-GlcNAc to the synthetic acceptor GlcNAcβ1,2Manα1,6Glcβ1-octyl per mg of protein as described.

L-PHA lectin and Western blotting: Proteins (50 µg per sample), separated on 8-16% gradient SDS-PAGE under reducing conditions, were transferred electrophoretically onto PVDF membrane. Western blots were performed with antibodies to phospho-MAPK (Thr202/Tyr204) and anti-Akt/PKB and phospho-Akt/PKB (New England Biolabs). For lectin blots, membranes were probed with 0.05 µg/ml L-PHA, followed by incubation with a rabbit anti-L-PHA antibody (1:1000) and with HRP-donkey anti-rabbit antibody (Amersham).
**Skin Inflammation:** DTH response was primed by applying 100 μl of 5% (w/v) 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) in ethanol/acetone (3:1, v/v) epicutaneously to the shaved backs of $Mgat5^+$ mice and littermate $Mgat5^{-/-}$ controls. Four days after sensitization, the mice were challenged by applying 25μl of 1% (w/v) oxazolone in olive oil/acetone (3:1, v/v) on each side of the right ear. As control, the left ear received 25 μl of olive oil/acetone on each side. Ear swelling was measured with a micrometer at 24 hr intervals for the next 5 days, and swelling was reported as the difference between the thickness of the right ear minus the thickness of the left ear. Results are reported for 7 $Mgat5^+$ mice and 6 $Mgat5^{-/-}$ mice. To investigate skin hypersensitivity, arachadonic acid (2 mg in 25 μl ethanol) was applied to the right ear of $Mgat5^+$ and $Mgat5^{-/-}$ littermate control mice. The left ear received 25 μl of ethanol alone. Ear swelling was measured with a micrometer at 2 hr intervals and was reported as above. Five mice of each genotype were used per experiment and the experiment was repeated twice.

**Induction of peritoneal exudates and cell adhesion:** To induce leukocyte extravasation, mice received an i.p. injection of thioglycollate broth (2 ml of 3% w/v), and 3 h later leukocytes were recovered from the peritoneal cavity by lavage and counted. The cells were immediately resuspended in alpha-MEM at $10^5$ cells/ml and applied in 100 μl aliquots to 96 well plates coated with serial dilutions of fibronectin. Neutrophil and T cell adhesion proceeded for 30 min, plates were washed with PBS and attached cells counted as described. To examine focal adhesions, embryonic fibroblasts were allowed to attach overnight to glass slides coated with 1 μg/ml of fibronectin in serum free alpha-MEM. The cells were stained with rhodamine-phalloidin, anti-paxillin antibodies (Transduction Laboratories), and Hoechst 33258 stain according to manufacturer's instructions. Fluorescence images of the cells were obtained using a deconvolution microscope and digital capture.
Stimulation of T cells in vitro: Spleen cells from 8-12 wk old mice were harvested and stimulated for 48 h as previously described (Kruisbeek, 1991). High seeding density was 2.5X\(10^5\) cells/well, and low seeding density was 5X\(10^4\) cells/well. Two \(\mu\)Ci of \(^3\)H-thymidine were added for the last 20 h of incubation, and cells were harvested on fiberglass filters and radioactivity was measured in a \(\beta\)-counter. T cells were purified (>90% pure) by panning on plates pre-coated with anti-CD19 antibody (Pharminen). Stimulatory lectins and antibodies used were L-PHA and Con A (Sigma), anti-CD3 antibody, clone 2C11 (Cedarlane) and anti-TCR antibody, clone H59.72 (generous gift of Dr. K. Siminovitch).

FACS analysis and TCR internalization: Single cell suspension of spleens, thymi and lymph nodes were stained with FITC- or PE-conjugated antibodies (Pharminen) reactive to CD3\(\epsilon\), TCR-\(\alpha/\beta\), CD4, CD8, CD69, B220, CD45 and CTLA-4. FITC-L-PHA (Sigma) was also used for analysis. Propidium iodide (5 \(\mu\)g/ml) was added to the cells to facilitate dead cell exclusion. Analysis was carried out on a FACSscalibur machine using FACSscan software. To measure TCR internalization, purified splenic T cells, stimulated with either 0.2 \(\mu\)g/ml anti-CD3 or 10 ng/ml PMA for varying lengths of time were harvested and stained with FITC-anti-TCR. PMA concentrations were not limiting as 10, 50 and 100 ng/ml produced similar internalization and cell activation results. LacZ activity in \(Mgat5^-\) T cells was detected by loading cells with fluorescin-di-\(\beta\)-D-galactopyranoside (FDG) (Molecular Probes) at 10\(^{\circ}\)C, and allowing the reaction to proceed for 30 min. The reaction was stopped by the addition of 1 mM phenyl-\(\beta\)-thiogalactoside, and analysis was carried out on a FACSscalibur machine.
CHAPTER 4: GlcNAc-TV Regulates Tumor Growth and Metastasis
INTRODUCTION

GlcNAc-TV is transcriptionally regulated by the Ras/MAPK/Ets pathway (Kang et al., 1996; Lu and Chaney, 1993) and its activity is commonly increased in human and rodent cancers. Studies on mutant tumor cell lines deficient in the enzyme, as well as overexpression studies of Mgat5, support the hypothesis that β1,6GlcNAc-branched N-glycans are positive effectors of tumor growth and metastatic potential (Dennis et al., 1987; Lu et al., 1994; Seberger and Chaney, 1999). Although experiments with somatic mutant tumor lines, and with revertant cell lines, have provided convincing evidence that β1,6GlcNAc-branched N-glycans are required for efficient tumor development and metastasis, some aspects of oncogenesis could not be explored using this approach. All previous studies on the role of GlcNAc-TV in tumor progression and metastasis were done with transplanted tumor cells, which were already fully transformed. Therefore, these experiments could not explore the role of GlcNAc-TV in tumor initiation, nor look at the effect of GlcNAc-TV deficiency in the host animal versus tumor.

In order to examine the effect of GlcNAc-TV on tumor formation and metastasis in a whole animal model, we crossed GlcNAc-TV deficient mice (Mgat5−/− mice) with mice transgenic for the polyoma middle T antigen under the regulation of the mammary specific MMTV promoter (PyMT). The PyMT antigen is a potent oncogene and its transforming activity is dependent on its ability to associate and activate c-Src, PI3K and Shc, which collaborate via intersecting signaling pathways to alter gene expression and cell behavior (Guy et al., 1998; Guy et al., 1994; Webster et al., 1998). Expression of the MMTV-PyMT transgene results in the widespread transformation of the mammary epithelium leading to multifocal mammary adenocarcinomas, and the majority of mice develop multiple metastases in the lung (Guy et al., 1992). The short latency period
between the expression of PyMT, which starts with sex steroid production during the maturation of the mammary gland, and widespread transformation, and the multifocal nature of the arising tumors suggest that the expression of PyMT alone is sufficient for tumorigenesis (Guy et al., 1992). Therefore, this model is attractive because the influence of heritable mutations on PyMT oncogenesis can be studied in vivo in a system where no additional mutations are necessary to produce tumors.

In this study, we show that GlcNAc-TV deficient mice bearing the PyMT transgene have a 1.5 fold extended latency period in forming palpable mammary tumors. These tumors also grow at a 6.7-fold slower rate than do tumors of wild type littermates, resulting in 4.5-fold reduction in tumor weight in late stages of the disease. GlcNAc-TV deficiency also resulted in >95% reduction in the incidence of lung metastases. By activating c-Src/FAK, Shc/Ras and PI3K, the PyMT protein contributes to the reorganization of cytoskeletal structures and turnover of focal adhesions. GlcNAc-TV deficient embryonic fibroblasts and primary PyMT-transformed tumor cells show reduced spreading and increased cortical stress fiber content when plated on fibronectin in serum free media. The results presented below suggest that GlcNAc-TV activity may enhance the formation and turnover of focal adhesions and thereby increase signaling via the integrin/PI3K/PKB pathway, thus enhancing cell proliferation, migration and metastatic potential.
RESULTS

Generation of PyMTMgat5−/− mice: Mgat5+/− females were crossed with a PyMT founder male to generate PyMTMgat5+/− mice. PyMTMgat5+/− males were crossed with Mgat5+/− females, and 42 PyMT female progeny were used in the experiments. A PCR strategy was employed to identify PyMT mice and to determine their Mgat5 genotype (Figure 1A). PyMT expression was similar in tumors from mice of all three genotypes, as determined by Northern analysis (Figure 1B).

Mammary tumors form with greater latency in Mgat5−/− mice: Mgat5+/+ and Mgat5+/− mice developed palpable tumors by the age of 8 weeks, but no palpable tumors appeared on Mgat5−/− mice until 12 weeks of age. Tumors were detected in all 10 mammary pads of Mgat5+/+ and Mgat5+/− mice by 20 weeks of age, but not until week 27 in Mgat5−/− mice (results summarized in Figure 2a). The time (T50) to 50% of the mammary glands developing palpable tumors was 14 weeks for Mgat5+/+ and Mgat5+/− mice, and 20.7 weeks for Mgat5−/− mice (a 1.5-fold delay). Mammary pad from Mgat5−/− mice developed normally, indicating that the effect of GlcNAc-TV deficiency on PyMT-mediated tumorigenesis is not due to altered mammary pad development. In addition, microscopic tumor nodules were observed in mammary pads of 19 week old mice of all Mgat5 genotypes. The number of tumor foci ranged from 0 to >100, and did not correlate with the Mgat5 genotypes. Thus, the multifocal nature of PyMT-dependent tumors is maintained on a GlcNAc-TV deficient background (Figure 2b).
FIGURE 1: Generation of $M{gat}^5{-}/PyMT^+$ mice and PyMT transgene expression.

(A) PCR was used to identify PyMT positive mice and to determine their $M{gat}^5$ genotype. PCR with PyMT specific primers amplifies a 530 bp fragment. The wild type allele of $M{gat}^5$ was detected by PCR with primers specific for the first coding exon of $M{gat}^5$, which generated a 446 bp fragment. The mutant allele was detected with primers specific for LacZ, which generated a 320 bp fragment. (B) Northern analysis of PyMT transgene expression. RNA was extracted from PyMT mammary tumors from $M{gat}^5{+/-}$, $M{gat}^5{+/-}$ and $M{gat}^5{-}$ mice and 10 µg were loaded per lane. The RNA was probed with a 1.5 kb cDNA fragment comprising the entire coding sequence of PyMT and the membrane was stained with methylene blue to visualize the 28S RNA band to ensure equivalent loading and transfer.
B

n h h w

PyMt

rRNA

A

PyMT

null

wild type
FIGURE 2: PyMT tumor development is delayed in $Mgat5^{-/-}$ mice.

(A) Littermates transgenic for PyMT on $Mgat5^{+/+}$, $Mgat5^{+/-}$ or $Mgat5^{-/-}$ background were palpated for the development of mammary tumors starting at 6 weeks of age. In total, 9 $Mgat5^{+/-}$, 17 $Mgat5^{+/-}$, and 14 $Mgat5^{-/-}$ PyMT+ female mice were examined. (B) wholemount preparations of the 4th mammary pad from 19 week old PyMT$Mgat5^{-/-}$ mice were stained with Carmine alum stain to visualize tumor microfoci prior to the formation of palpable tumors. Multiple foci were observed in these mammary pads, and no apparent correlation was observed between the number of foci per mammary pad and $Mgat5$ genotype, when 4 $Mgat5^{+/-}$, 6 $Mgat5^{+/-}$ and 4 $Mgat5^{-/-}$ mammary fat pads were compared.
**Tumor growth is slowed in Mgat5−/− mice:** In order to compare the rate of tumor growth in Mgat5−/− mice to Mgat5+/− and Mgat5+/+ mice, palpable tumors were measured weekly and the tumor area was plotted (Figure 3A). Tumors in Mgat5−/− mice grew at a 6.7 fold slower rate than did tumors in Mgat5+/− or Mgat5+/+ mice. The slower growth rate was reflected in a 4.5 fold decrease in tumor weight at 26 week old mice (Figure 3B). Mgat5−/− mice had 3.4 ± 0.8 g of tumor as compared to the Mgat5+/− and Mgat5+/+ mice which averaged 15.1 ± 1.8 g and 13 ± 2.8 g, respectively. A small subset of GlcNAc-TV deficient tumors (5/140, 3.7%) grew slowly initially, but had a sharp increase in their growth rate to resemble Mgat5+/− and Mgat5+/+ tumor growth 3-4 weeks after tumors became palpable (Figure 3C). Mammary pads from 26 week old GlcNAc-TV deficient mice displayed a range of histopathology, including hyperplasia, non invasive dysplasia and invasive carcinoma with necrosis (Figure 4A,B). In contrast, All mammary pads from 26 week old Mgat5+/+ and Mgat5+/− mice had tumors that were without exception invasive, undifferentiated and with central areas of necrosis (Figure 4C). Hyperplasia is defined as intraluminal proliferation of cells, resulting in a duct wall that is more than one cell thick. Dysplasia is defined as the loss of the uniformity of individual cells, as well as their architectural orientation. Malignant tumors show increased nuclear content and an irregular, invasive growth through basement membrane.

Adjacent sections to the hematoxylin and eosin stained sections were immunostained to quantify apoptosis and proliferation. Klenow was used to label broken DNA fragments in apoptotic cells, and a monoclonal antibody was used to detect proliferating cell nuclear antigen (PCNA) in cycling cells. The percentage of apoptotic cells was not significantly different between genotypes (Table 1). However, proliferation was significantly reduced in Mgat5−/− tumors (Figure 5A-D) in all stages of tumorigenesis.
FIGURE 3: GlcNAc-TV deficiency causes slower tumor growth.

(A) Palpable mammary tumors on mice of all Mgst5 genetic backgrounds were measured weekly with calipers, and the average of two measurements / tumor / week was plotted. In total 50 Mgst5+/+, 50 Mgst5+/ and 50 Mgst5−/− tumors were measured. Fast growing tumors were not included in the Mgst5−/− category. (B) PyMT-transgenic mice were weighed at 26 weeks and the weight of a littermate female not bearing the PyMT transgene was subtracted. 11 Mgst5+/+, 15 Mgst5+/− and 14 Mgst5−/− were weighed. (C) A small subset of GlcNAc-TV deficient tumors (5/140, 3.6%) displayed an altered growth rate 3-4 weeks after they were first detected by palpation. The growth of three such tumors are plotted.
FIGURE 4: Histopathology of mammary fat pads and tumors in 26 week old PyMTMgat5−/− and PyMTMgat5+/− mice.

Tumors from Mgat5−/− (A,B) and Mgat5+/− (C) were paraffin-embedded and 8 micron sections were stained with hematoxylin and eosin. h, hyperplasia; d, dysplasia; t, tumor; n, necrotic area.
FIGURE 5: Proliferative index in mammary fat pads and tumors in PyMTMgat5+/− and PyMTMgat5−/− mice.

Dysplastic epithelia (A,B) and invasive carcinomas (C,D) from Mgat5+/− (A,C) and Mgat5−/− (B,D) were subjected to immunohistochemistry with an antibody against the proliferating cellular nuclear antigen (PCNA) to visualize proliferating cells. (E) A significant difference (p<0.05) was observed in the proliferative index in all stages of transformation between the two genotypes.
PCNA positive cells (%)
Table 1: Number of apoptotic cells per 10X magnification view.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>+/−</th>
<th>−/−</th>
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</thead>
<tbody>
<tr>
<td>Pathological stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysplasia</td>
<td>4 ± 2</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>Invasive tumor</td>
<td>17 ± 10</td>
<td>16 ± 8</td>
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Tumors from $Mgat5^{+/−}$ and $Mgat5^{−/−}$ mice were paraffin embedded and sections were labeled with Klenow to visualize apoptotic cells. Ten random fields / mouse were scored, and tumors from two mice per genotype were examined.
**Expression from the Mgat5 locus is enhanced with PyMT transformation and tumor progression:** The mutant allele of Mgat5 was generated by replacing the coding portion of the first coding exon with the bacterial reporter gene LacZ, encoding β-galactosidase. Thus, expression of LacZ is under the control of the Mgat5 promoter, and reflects the tissue distribution observed for Mgat5 transcripts by RNA *in situ* hybridization (Granovsky et al., 1995). Mammary fat pads from Mgat5+/− mice were stained with x-gal to visualize β-galactosidase expression in tumors of different sizes and apparent stages of progression. β-galactosidase activity was not evident in normal mammary epithelium, but activity was detected in tumors (Figure 6A). Uniformly intense β-galactosidase activity was observed in Mgat5+/− tumors. However, in Mgat5/− tumors, intense staining was observed focally, while the majority of the tumor area was weakly stained (Figure 6B). LacZ expression was uniform and high in 3/3 fast growing Mgat5/− tumors, comparable to that of Mgat5+/− tumors (Figure 6C).

**GlcNAc-TV deficiency suppresses metastasis:** Mice transgenic for PyMT develop metastatic disease in lung, and GlcNAc-TV activity and the β1,6GlcNAc-branched N-glycans has been causally implicated in increased metastatic potential. The numbers of surface lung metastases were compared across the Mgat5 genotypes (Figure 7A). A 20-fold reduction was observed in the number of metastases in Mgat5+ mice compared to Mgat5−/− and Mgat5−/− controls. When the number of metastatic nodules was normalized to tumor weight per animal, an 11-fold reduction was observed in Mgat5+ mice compared to Mgat5−/− and Mgat5−/− controls (Figure 7B). In addition, the average size of the metastatic nodules was smaller in the Mgat5+ mice. Microscopic examination of lungs from 6 Mgat5+ mice revealed no microscopic tumor foci.
FIGURE 6: Lac Z expression in \( M{gat}^{5+/−} \) and \( M{gat}^{5−/−} \) tumors.

(A) Whole mammary pads from 14 week old \( M{gat}^{5+/−} \) mice were stained for \( \beta \)-galactosidase activity. Staining was not observed in premalignant mammary fat pads, but intense staining was observed in tumor foci (arrows). (B,C) Tumors were resected from 26 week old \( M{gat}^{5−/−} \) mice and stained for \( \beta \)-galactosidase activity. The majority of \( M{gat}^{5−/−} \) tumors exhibited punctate x-gal staining (B), but fast growing tumors (C) stained intensely and uniformly. lac Z, foci of x-gal staining; n, necrosis.
FIGURE 7: GlcNAc-TV activity is required for metastasis.

(A) *Mgat5*+/+ and *Mgat5*−/− mice were sacrificed when tumor burden reached ½ body weight, or when bleeding from ulcerated tumors was observed, and their lungs were resected and visually examined for surface metastatic foci. *Mgat5*−/− were sacrificed at the same time as their *Mgat5*+/+ and *Mgat5*−/− littermates (26-28 weeks, denoted by black circles). White circles indicate the number of metastatic foci on lungs from mice sacrificed at 19 weeks of age with low tumor burden and with no evidence of ulcerated tumors. White squares indicate the number of metastatic foci on lungs from *Mgat5*−/− mice with fast-growing tumors. (B) The number of metastatic foci was normalized to the tumor weight of the animal.
**A**

Lung metastases

\[\begin{array}{c}
Mgat5: \\
+/- \\
+/- \\
-/- \\
\end{array}\]

○ Mice sacrificed at 19 weeks

□ Mgat5-/- mice with fast growing tumors

**B**

Metastatic foci / g tumor

\[\begin{array}{c}
Mgat5: \\
+/- \\
+/- \\
-/- \\
\end{array}\]

+/

\(6.118\)

+/

\(5.020\)

-/

\(0.368\)

\(\star\) \(p<0.0005\)
The number of metastatic foci was normalized to the tumor weight of the animal. The average number of metastatic foci / g tumor was 0.37±0.14 in $Mgat5^{-/-}$ mice, compared to 5.0±0.9 and 6.1±1.9 foci / g tumor in $Mgat5^{+/+}$ and $Mgat5^{+/-}$ mice, respectively. Tumor burden on the lungs often results in cardiac hypertrophy, a physiological response to poor lung capacity. Cardiac hypertrophy was scored in 13/27 $Mgat5^{+/-}$ and $Mgat5^{+/-}$ mice, but in 0/15 $Mgat5^{-/-}$ mice.

$Mgat5^{-/-}$ PyMT tumor cells display aberrant cytoskeletal architecture: In order to investigate the effect of GlcNac-TV deficiency on cell adhesion and cytoskeletal morphology, PyMT tumor cells were plated on fibronectin-coated cover slips in serum free media, and subsequently stained with the nuclear stain Hoechst33258, actin-specific rhodamine-phalloidin and FITC-anti-paxillin antibody (Figure 8A-D). The PyMT$Mgat5^{-/-}$ (Figure 8B) tumor cells showed impaired membrane ruffling and a dispersed and punctate distribution of focal adhesions compared to PyMT$Mgat5^{+/-}$ tumor cells (Figure 8A). The PyMT$Mgat5^{-/-}$ tumor cells had denser actin fibers with very little paxillin localized to putative attachment sites at the edges of the cell. Paxillin binds β1 integrin, as well as signaling molecules FAK, Csk and c-Src at discrete sites of cell attachment in spreading and motile cells. Membrane ruffling and filopodia formation require PI3K activation. The pattern of actin stress fibers in $Mgat5^{+/-}$ tumor cells treated with wortmannin, a PI3K inhibitor, was similar to that of $Mgat5^{-/-}$ tumor cells, with heavy cortical actin fibers replacing the fine actin filaments of $Mgat5^{+/-}$ cells cultured in the absence of wortmannin (Figure 8A and C). Cell spreading and membrane ruffling induced
FIGURE 8. Effects of GlcNAc-TV deficiency on intracellular cytoskeletal architecture.

*Mgat5*+/-(A) and *Mgat5*-/-(B) PyMT tumor cells were plated on fibronectin and stained with the nuclear stain Hoechst322, actin-specific rhodamine-phalloidin and FITC-anti-paxillin antibody. Fine actin filaments and ruffling were observed in *Mgat5*+/+ tumor cells, characteristic of motile cells. In contrast, *Mgat5*-- tumor cells exhibited morphology characteristic of stationary cells, with cortical actin stress fibers establishing the perimeter of the cell.

(C) The staining pattern of *Mgat5*+/+ tumor cells incubated with wortmannin was similar to that of *Mgat5*-- cells, with heavy actin stress fibers replacing the fine actin filaments of *Mgat5*+/+ cells. (D) Cells from a fast-growing *Mgat5*-- tumor were stained for actin and paxillin. The cell population showed considerable heterogeneity.
by PyMT in serum free conditions is impaired in PyMT<sup>Mt5<sup>- tumor cells, suggesting that PyMT-induced activation of focal adhesion signaling is reduced in PyMT<sup>Mt5<sup>- tumor cells.

Cells from a fast-growing <i>Mgat5<sup>-</i> tumor were also plated on fibronectin and stained for actin and paxillin (Figure 8D). Greater morphological heterogeneity was observed in these cells than in slow-growing <i>Mgat5<sup>-</i> cells. Cells with filapodia, focal adhesions and actin fibers, as well as cells with focal adhesions but little actin filaments were observed. This mixed cell population may reflect tumor progression and evolution of the population towards a phenotype similar to that of the <i>Mgat5<sup>+</i>-</i> phenotype.

**Wild-type ES cells form tumors in <i>Mgat5<sup>-</sup></i> mice:** Subcutaneously injected ES cells form teratocarcinomas in syngeneic mice. GlcNAc-TV-dependent glycosylation affects both host and tumor glycoproteins, and therefore a host effect on tumor growth could not be ruled out. In order to investigate the contribution of host GlcNAc-TV on tumor formation and growth, we injected wild-type ES cells into <i>Mgat5<sup>+</sup></i><sup>/+</sup>, <i>Mgat5<sup>-</sup></i><sup>/+</sup> and <i>Mgat5<sup>-</sup></i><sup>-</sup> mice. Tumors formed in all <i>Mgat5<sup>+</sup></i><sup>/+</sup> and <i>Mgat5<sup>-</sup></i><sup>-</sup> animals, demonstrating that <i>Mgat5<sup>-</sup></i> animals do not mount a significant anti-tumor immune response against cells with β1,6GlcNAc-branched glycans. However, the kinetics of tumor formation and growth were significantly different in <i>Mgat5<sup>+</sup></i><sup>/+</sup> and <i>Mgat5<sup>-</sup></i><sup>-</sup> mice (Figure 9). Formation of tumors was delayed in <i>Mgat5<sup>-</sup></i> mice until week 6 compared to week 3 for wild type mice, but once detected, tumors grew significantly faster than tumors in <i>Mgat5<sup>+</sup></i><sup>/+</sup> mice. Surprisingly, tumors did not grow in 4/4 <i>Mgat5<sup>+</sup></i><sup>-</sup>, a result requiring further investigation. These findings suggest that glycosylation of glycoproteins in the host microenvironment of the tumor plays a significant role in tumor progression.
FIGURE 9: GlcNAc-TV deficiency in host animals affects growth characteristics of wild type ES cell-derived teratocarcinomas.

2X10⁶ wild-type ES cells were injected s.c. into the left hind leg of $Mgat5^{-/-}$, $Mgat5^{+/}$ and $Mgat5^{+/+}$ mice. Mice were palpated weekly for the formation of tumors, and the diameter of palpable tumors was measured weekly. Tumors formed on 5/5 $Mgat5^{-/-}$, 0/4 $Mgat5^{+/}$ and 4/4 $Mgat5^{+/+}$ mice. The graph represents growth measurements of 4/5 $Mgat5^{-/-}$, 4/4 $Mgat5^{+/}$ and 3/4 $Mgat5^{+/+}$ mice. One $Mgat5^{-/-}$ mouse and 1 $Mgat5^{+/+}$ mouse developed ascites tumors and were not included in the growth measurements.


**DISCUSSION**

*GlcNAc-TV deficiency delays but does not inhibit tumorigenesis:* GlcNAc-TV activity has been causally implicated in tumor growth and metastasis (Dennis et al., 1987; Lu et al., 1994; Seberger and Chaney, 1999). To elucidate the role of GlcNAc-TV in these processes, we generated a GlcNAc-TV deficient mouse and crossed it with a transgenic mouse expressing the PyMT antigen under the control of a mammary-specific promoter. PyMT is a potent oncogene, which becomes tyrosine phosphorylated and serves as a docking protein for membrane localization and activation of Src kinases, PI3K and the Shc adaptor protein, all of which contribute to transformation (Blaikie et al., 1997; Webster et al., 1998; Yi and Freund, 1998). PyMT is sufficient to transform immortalized cells in culture, and the MMTV-controlled transgene induces multifocal tumorigenesis in breast epithelium (Dilworth et al., 1994; Guy et al., 1998).

Mice of all *Mgat5* genotypes developed invasive mammary tumors in 10/10 mammary pads by 26 weeks of age. Multiple tumor foci were detected in 19 week old mice, even in mammary fat pads that lacked palpable tumors. The number of foci was variable with no significant difference amongst the genotypes in the small number of samples examined. The high incidence of *in situ* carcinomas and the development of multiple tumors per mouse and per fat pad indicate that PyMT-induced multifocal transformation occurs in *Mgat5* mice, but growth of the tumors is delayed markedly.

The PyMT oncogene activates two main pathways that collaborate to allow the formation of tumors. The Ras/MAPK pathway is activated by the association of PyMT with Shc and Src, and the PI3K/PKB pathway is activated by engagement of PI3K (Guy et al., 1994; Meili et al., 1998; Webster et al., 1998). In contrast to wild type mice carrying the MMTV-PyMT transgene, c-Src deficient mice rarely developed mammary
tumors, suggesting that c-Src is essential for PyMT mediated transformation (Guy et al., 1994). Src activation is commonly increased in human breast tumors making the study of Src as relevant a model to the human disease (Verbeek BS et al., 1999), as the study of mutations affecting the Ras and the PI3K/PKB pathways, also common in human tumors (Velders and Kast, 1999). However, Src is not sufficient for transformation, as mice transgenic for activated c-Src develop hyperplasia but no tumors (Webster et al., 1995). Muller’s group has generated two transgenic mouse lines carrying mutant PyMT antigens that are unable to associate with either PI3K or Shc (Webster et al., 1998). Both mutations attenuated the delay and reduced tumorigenesis. The presence of either mutant was sufficient for the formation of global mammary epithelial hyperplasia, which progressed to the formation of focal mammary tumors with a longer latency than observed in mice transgenic for wild type PyMT. Consistently, both mutations in PyMT caused a 2.3-2.7 fold delay before palpable tumors were formed. In mice transgenic for either PyMT mutant, the transition from hyperplasia to tumor was commonly associated with a secondary mutation. A second mutation in the PyMT gene, restoring the ability of PyMT to bind Shc, was observed in a subset of tumors arising in mice carrying the Shc binding mutant of PyMT. Tumors in mice transgenic for either PyMT mutant showed increased expression of ErbB-2 and ErbB-3 receptor tyrosine kinases, which recruit and activate PI3K and Shc in a ligand dependent manner (Webster et al., 1998). The phenotype of the \textit{Mgat5}^{−/−} mice was different from that of the mice transgenic for either mutant in several respects. Palpable tumors arose earlier in the \textit{Mgat5}^{−/−} mice than in either of the PyMT mutants, which were delayed significantly longer. As well, the multifocal nature of the tumors in \textit{Mgat5}^{−/−} mice was similar to the phenotype of the tumors arising in mice transgenic for wild type PyMT. Thus, we concluded that GlcNAc-
TV activity is not critical to PyMT-dependent tumorigenesis, but rather, significantly retards tumor growth.

**GlcNAc-TV deficiency slows tumor growth:** Although Mgat5+ mice developed tumors in all mammary pads examined, there was a significant lag in the formation of palpable tumors. The delay observed on the Mgat5+ genetic background is comparable to that observed for PyMT-mediated tumors in Grb2+/− and Ets-2+/− mice (Cheng et al., 1999) (personal communication, R. Oshima). Grb2 is an adaptor protein, which mediates Ras activation by Shc and by receptor tyrosine kinases (Cheng et al., 1999). Ets-2 is a transcription factor positioned downstream of Ras, which is required for expression of MMPs and VEGF (Wasylyk et al., 1998). The promoter of Mgat5 contains AP1, AP2 and PEA3/ets sites (Saito et al., 1995), and Mgat5 is activated by oncogenes in the Ras pathway (Kang et al., 1996; Chen et al., 1998). The slower development of tumors in Mgat5+ mice is consistent with the observed phenotypes of mice bearing other mutations in the Ras pathway. Therefore, GlcNAc-TV may be a downstream effector of PyMT-mediated transformation and metastasis, in a manner similar to downstream effectors of Ras signaling.

VanderElst and Dennis have previously shown that reduced branching of N-glycans on the surface of MDAY-D2 mutant tumor cells resulted in slower cellular growth rates *in vitro* and in differences in tumor weight *in vivo* (VanderElst and Dennis, 1991). Interestingly, relatively small differences in the measured doubling time of the cells *in vitro* (12%) resulted in more than doubling of the tumor size *in vivo* over 3 weeks. Mgat5−/− cells proliferate significantly less than Mgat5+/+ cells in all stages of transformation (see Figure 6E), as measured by PCNA immunohistochemistry. Mgat5−/− mice have a reduced proliferative index by 2-fold in hyperplastic epithelium, by 1.5-fold in dysplastic epithelium, and by 1.1 fold in invasive tumors, compared to Mgat5+/+ mice. All differences were statistically significant. These differences in proliferation are
sufficient to account for the 6-fold difference observed in the growth rate of tumor area and the 4.5 difference in tumor weight. Indeed, if all other conditions were the same, a 4.4% difference in cellular doubling times would be sufficient to account for a 4.5 fold difference in tumor weight over the period of 7 weeks, which is the period used in this experiment.

**Mgat5 expression is a marker of tumor progression:** Analysis of LacZ reporter gene expression in Mgat5<sup>−/−</sup> and Mgat5<sup>+/−</sup> mammary fat pads showed that there are 3 relative levels of β-galactosidase activity in these tissues. Normal and hyperplastic mammary epithelium of Mgat5<sup>−/−</sup> mice did not express LacZ, but intense staining was observed in tumors from these mice. The majority of tumors from Mgat5<sup>−/−</sup> mice (96%) grew more slowly than tumors from Mgat5<sup>−/−</sup> or Mgat5<sup>+/−</sup> mice, and LacZ expression was low, with only sporadic foci of intense staining. However, in the fast-growing Mgat5<sup>−/−</sup> tumors lacZ expression was comparable to that of Mgat5<sup>−/−</sup> tumors. This suggests a secondary mutation or epigenetic event has occurred in these PyMT tumors and provided both a growth advantage and restores Mgat5 promoter activity to that of the Mgat5<sup>−/−</sup> or wild type PyMT tumors. The staining pattern indicates that GlcNAc-TV activity and its products provide positive feedback for optimal signaling leading to expression from the Mgat5 locus.

* formula for calculating the relationship between tumor weight and cellular proliferation ratios:  
\[
\frac{\text{Mgat5}^{−/−}\text{tumor weight}}{\text{Mgat5}^{+/−}\text{tumor weight}} = \left(\frac{e^{(\ln 2)\Delta g}}{e^{(\ln 2)\Delta g}}\right)
\]  
where \(\Delta\) is the fold difference in cellular proliferation (VanderElst and Dennis, 1991).
Optimal expression is restored in the fast-growing \(Mgat5^{-/-}\) tumors, suggesting that the restoration of fast growth and \(Mgat5\) expression require signaling through the same pathway. Lac Z expression is not observed in normal or hyperplastic mammary tissues, indicating that transcription from the \(Mgat5\) locus is turned on after PyMT induced transformation. This staining pattern further supports the model that \(\text{GlcNAc-TV} \) activity functions post transformation to influence the rate of tumor growth, but is not required for tumor formation.

**GlcNAc-TV activity is required for efficient metastasis and might be necessary for optimal PyMT-induced PI3K signaling:** Transfection of \(Mgat5\) into metastatic mammary tumor cells enhances their metastatic potential 4-40 fold (Lu et al., 1994). In the current study, GlcNAc-TV deficiency resulted in >95% reduction in lung metastases. This observation held true when the number of metastatic foci was normalized to the weight of the primary tumors per animal and in \(Mgat5^{-/-}\) mice with fast-growing tumors. Therefore, it is possible that metastatic potential and tumor growth rates are separate and distinct phenotypic features of the \(Mgat5^{-/-}\) mouse. The distinction between tumor growth and metastatic potential has been previously observed in plasminogen deficient mice (Bugge et al., 1999). Plasminogen is a protease that efficiently degrades fibrin, thus participating in ECM remodeling. Expression of the PyMT transgene in plasminogen deficient mice resulted in the development of mammary tumors indistinguishable from those developed by wild type littermates. However, a 6-fold reduction in lung metastases was observed in plasminogen-deficient mice (Bugge et al., 1999).

The abrogation of metastasis in \(Mgat5^{-/-}\) mice might be due to changes in the ability of \(Mgat5^{-/-}\) tumor cells to migrate on ECM. ECM-dependent proliferation and migration is mediated primarily by the integrins, a family of cell surface heterodimeric receptors. Integrins are comprised of extracellular domains that recognize specific ECM
proteins, and cytoplasmic domains, which associate with bundles of actin microfilaments and affiliated structural proteins, such as vinculin. Integrin subunits α5, αv, and β1 have been previously shown to be substrates for GlcNAc-TV-dependent glycosylation (Demetriou et al., 1995). These subunits form VLA-5, the cellular fibronectin receptor (Ruoslahti, 1996). Mgal5-transfected MvLu1 cells are less adherent to fibronectin and are more migratory than the parental cell line (Demetriou et al., 1995), supporting the hypothesis that β1,6GlcNAc-branching of N-glycans may regulate cellular adhesion and migration.

As was shown in Chapter 3 (Figure 6) and in this chapter, the cytoskeletal organization of Mgal5+/− fibroblasts and PyMT tumor cells differs from wild type and Mgal5−/− cells. In addition, resting Mgal5−/− T cells have higher content of actin stress fibers than do wild type cells. These observations suggest that Mgal5−/− cells are more stationary, and that adhesion-dependent signaling requires β1,6GlcNAc-branched N-glycans. In Mgal5−/− embryonic fibroblasts, paxillin, a downstream effector of Src activation by integrin signaling, was not localized to focal adhesions, in contrast to Mgal5+/− cells. The failure of Mgal5−/− fibroblasts to properly localize paxillin indicates that GlcNAc-TV deficiency inhibits focal adhesion formation and turnover required for cell spreading and motility. Paxillin is a multi-domain molecule with binding sites for both structural proteins and signaling proteins involved in cell motility and cell proliferation (Turner, 1998). Upon cellular adhesion to ECM, paxillin is targeted to focal adhesions by an as yet undefined recruiting molecule, where it interacts with the focal adhesion proteins vinculin and talin, and where it is phosphorylated by FAK and Src. The phosphorylation of paxillin results in the formation of SH2 domains which serve as docking sites for signaling proteins such as Crk, thus contributing to signaling through the Ras pathway. The physical features of paxillin suggest an important role for this
protein in recruiting common signal cascade components into close proximity with one another, thereby allowing efficient signal transduction through both cell proliferation and cell motility cascades (Turner, 1998). Thus, the lack of paxillin localization to putative focal adhesions in GlcNAc-TV deficient fibroblasts may result in inefficient signaling through Ras/MAPK and PI3K/PKB pathways.

Serum starved GlcNAc-TV deficient fibroblasts and resting T cells had similar levels of MAPK phosphorylation to wild type cells, but had reduced PKB phosphorylation. This finding suggests that signaling downstream of Ras was intact in GlcNAc-TV deficient cells, but that signaling through PI3K may have been inhibited. PI3K catalyzes the phosphorylation of inositol lipids at the 3' position on the inositol ring, resulting in the formation of 3-phosphorylated phosphoinositides (PtdIns) (Leevers et al., 1999). Phosphorylated PtdIns are second messenger molecules that participate in the transduction of mitogenic signals from activated tyrosine kinase receptors and of signals mediated by integrin engagement. Phosphorylated PtdIns are necessary for the activation of PKB. The binding of PtdIns(3,4)P₂ to the plextrin homology (PH) domain of PKB results in the translocation of PKB to the plasma membrane where it is phosphorylated by phosphoinositide-dependent Thr/Ser kinases. Therefore, PKB phosphorylation is a measure of PI3K activity (Leevers et al., 1999; Wymann and Pirola, 1998).

PI3K activity transduces signals downstream of receptor tyrosine kinases as well as engaged integrins. The addition of serum to cultured Mgat5⁻/⁻ fibroblasts, and the mitogenic stimulation of Mgat5⁻/⁻ T cells resulted in normal PKB phosphorylation, indicating that stimulation of PI3K activity through receptor tyrosine kinases was intact. However, phosphorylation was reduced in Mgat5⁻/⁻ cells in serum free conditions, consistent with the cytoskeletal morphology of the cells, and confirming than integrin signaling is compromised.
FIGURE 10: The effect of GlcNAc-TV deficiency on MAPK and PKB phosphorylation.

### A

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<th>Time (min)</th>
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<th>GlcNAc-TV</th>
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<td>5</td>
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<td>15</td>
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- anti-P-S473 PKB
- anti-PKB
- anti-T202/Y204 MAPK

### B

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<td>PKB</td>
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PyMT binds and activates Src and PI3K, and therefore it is possible that tumor cells are less dependent on integrin engagement to initiate signaling cascades leading to cell motility, as indicated by the translocation of paxillin to focal adhesions. However, our results suggest that PyMT tumor cells remain at least partially dependent on focal adhesion turnover, requiring GlcNAc-TV activity. In PyMT transformed cells, paxillin localized to focal adhesions in cells of all genotypes (this chapter, Figure 9). However, \textit{Mgat5}\textsuperscript{-/-} cells failed to undergo membrane ruffling, a necessary step for cell motility. Rather, \textit{PyMTMgat5}\textsuperscript{-/-} cells maintained heavy actin stress fibers suggesting little turnover of actin and focal adhesions. In contrast, the \textit{PyMTMgat5}\textsuperscript{-/-} tumor cells show membrane ruffling and fine actin filaments. A recent study suggests that paxillin phosphorylation in response to integrin engagement is partly dependent on PI3K activity. We have observed that \textit{Mgat5}\textsuperscript{-/-} tumors have hypophosphorylated PKB, and preliminary immunoprecipitation experiments show reduced phosphorylation of paxillin as well. These results indicate that PI3K activity is inhibited in \textit{Mgat5}\textsuperscript{-/-} tumors similarly to the inhibition observed in \textit{Mgat5}\textsuperscript{-/-} quiescent fibroblasts and T-cells.

The lack of membrane ruffling and the hypophosphorylation of PKB in \textit{PyMTMgat5}\textsuperscript{-/-} tumor cells suggest that signaling by the Rac and Cdc42 GTPases may be impaired. Membrane ruffling requires the activation of Cdc42 downstream of integrin engagement. Activated Cdc42 associates with PI3K and stimulates its activity (Reif et al., 1996), which, in turn, is required for the activation of Rac. PI3K-dependent activation of Rac results in membrane ruffling (Aspenstrom, 1999). Fast growing \textit{PyMTMgat5}\textsuperscript{-/-} tumors have comparable PKB phosphorylation levels to wild type PyMT tumors, and their growth rate is similar to wild type tumors as well. However, cells from the fast growing \textit{PyMTMgat5}\textsuperscript{-/-} tumors still exhibit considerably less membrane ruffling than wild type PyMT tumor cells do, indicating that the altered growth and cytoskeletal
rearrangement are separate and distinct characteristics of the \textit{Mgat5}^\text{-} tumor cell phenotype. These findings suggest that integrin signaling leading to Cdc42 activation may remain impaired in the fast growing PyMT\textit{Mgat5}^\text{-} tumors, even though a secondary mutation restored PI3K activity to wild type levels, and restores wild type-like growth rates.

\textit{Fast-growing Mgat5}^\text{-} tumors may be the result of a somatic mutation: The infrequent appearance of fast-growing \textit{Mgat5}^\text{-} tumors, which appear to arise from slow growing \textit{Mgat5}^\text{-} tumors, suggests the occurrence of rare mutations. The effect of such mutations is to release cells from the growth inhibition imposed by GlcNAc-TV deficiency. As in the case of the mice transgenic for mutant PyMT, one such possible secondary mutation would be the upregulation of the ErbB receptors, which are known to enhance PI3K activity and Shc phosphorylation. Such a scenario may also enhance Ras signaling, and therefore result in enhanced expression from the \textit{Mgat5} locus in an Ets-2 and AP1 dependent manner. The upregulation of LacZ expression in the fast growing tumors is consistent with this model.

The fast-growing \textit{Mgat5}^\text{-} tumors do not give rise to a higher incidence of metastases. This observation suggests that metastatic potential and tumor growth rates are separate and distinct phenotypic features of the \textit{Mgat5}^\text{-} mouse. Therefore, it is possible that the secondary mutation, sustained by the cells giving rise to fast-growing tumors, is sufficient to compensate for the proliferative deficiency but not for the metastatic deficiency caused by \textit{Mgat5} ablation. An amplification of intracellular signaling may drive proliferation and survival, while the deficiency in $\beta$1,6GlcNAc-branched N-glycans continues to restrict focal adhesion turnover and thereby physical movement of the tumor cells required for invasion and metastasis.

\textit{Autonomous vs host-dependent factors in tumor progression:} Glycosylation can
potentially affect tumor growth in both tumor-autonomous and host-dependent manner. Wild type ES cells were injected s.c. into mice of all Mtat5 genotypes to investigate the role of host GlcNAc-TV activity on tumor formation and growth. ES cells injected into Mtat5/− and Mtat5+/+ mice produced tumors in 100% of the mice. However, surprisingly, none of the Mtat5+/− mice developed tumors. Latency was longer in Mtat5/− mice, but once tumors formed, their growth rate was significantly greater, suggesting that host factors depend on GlcNAc-TV activity for tumor growth. A similar dependence on proper glycosylation was noted in GlcNAc-TIII deficient mice. Hepatocarcinomas resulting from diethylnitrosamine treatment progress more slowly in Mtat3/− mice compared to wild type littermates. However, Mtat3 is not expressed in mouse hepatocarcinoma cells, suggesting that a host paracrine effect, such as the full function of a growth factor, or a growth factor receptor, is dependent upon GlcNAc-TIII glycosylation to promote tumor growth (Bhaumik et al., 1998).

This experiment suggests that GlcNAc-TV deficiency in the host exerts an opposite effect on tumor growth rates than GlcNAc-TV deficiency of tumor cells. In addition, the differences in latency and growth rates between tumors in Mtat5+/+ and Mtat5−/− mice suggest that GlcNAc-TV may regulate more than one aspect of host permissiveness for tumor progression. Obvious candidates for further investigation include host immune responses to the ES cells, and the angiogenic potential of the Mtat5−/− mice.
METHODS

**Generation of Mgat<sup>+/</sup>PyMT<sup>+</sup> mice:** Mgat<sup>5/5</sup> mice were generated as described in Chapter 3. Mgat<sup>5/5</sup> female mice on 129/Sv background were mated with a male mouse transgenic for MMTV-PyMT on FVB background. Offspring were screened for the presence of the PyMT gene and their Mgat5 genotype was identified by PCR. PCR primers for PyMT were 5'GGAAAGCAAGTACTTCACAAGGG3' and 5'GGAAAGTCAGGAGCAGGG3' and generated a 530 bp fragment. For Mgat5 genotyping, the primer pair 5'GTAAGGACTCACAGCTGAGG3' and 5'GCCAAGGGGAATGGTACATTGC3' was used to amplify a wild type-specific fragment and the primer pair 5'CCCATCTACACCAACGTAACC3' and 5'CGCCACATATCCTGTCTTCC3' was used to amplify a mutant-specific fragment. The wild-type primers amplified a 446 bp fragment and the mutant primers amplified a 320 bp fragment.

**Northern analysis of PyMT expression:** Mice bearing mammary tumors were sacrificed and the tumors were snap frozen. RNA was isolated using Trizol (Gibco-BRL) according to manufacturer’s instructors. RNA was size fractionated on a 1% formamide-agarose gel and blotted onto Genescreen membrane. Radiolabeled PyMT cDNA (1.5 kb) containing the entire coding region, was used as probe.

**Detection and measurement of tumors and metastatic foci:** Tumors were detected by palpating each mammary fat pad weekly from the age of 6 weeks. Once tumors became palpable, two measurements / tumor / week were done using calipers. Mice were sacrificed when tumor burden reached 50% of body weight, or when tumors became ulcerated, and lungs were resected and surface metastatic foci were counted visually.

**Histological examination of tumors:** Tumors were fixed in 10% phosphate-buffered...
formalin, blocked in parafin, sectioned at 8 μm and stained with hematoxylin and eosin. Wholemount preparations of mammary fat pads were done as previously described. Briefly, the fat pads were spread on a glass slide and air-dried. The slides were fixed overnight in Carnoy’s solution (75% ethanol, 25% glacial acetic acid) and stained with Carmine alum stain (0.2% w/v Carmine dye, 0.5% w/v aluminum). Staining of sections for apoptosis by Klenow labeling of fragmented DNA and for proliferation by PCNA immunohistochemistry, were done as previously described (Fata et al., 1999).

Detection of β-galactosidase activity:

Mammary fat pads were fixed in 0.2% glutaraldehyde for 30 minutes, washed in PBS, and incubated overnight with X-gal staining solution (1mg/ml 4-chloro-5-bromo-3-indolyl-β-galactoside(X-gal), 4mM k$_4$Fe(CN)$_6$·3H$_2$O, 4mM K$_3$Fe(CN)$_6$, 2mM MgCl$_2$, 0.01% deoxycholate, 0.02% Nonidet-P40 in 0.1M sodium phosphate pH7.3). After staining, tumors were further fixed in 10% phosphate-buffered formalin.

**Immunofluorescent microscopy:** To examine focal adhesions, embryonic fibroblasts and primary tumor cells were allowed to attach overnight to glass slides coated with 1 μg/ml of fibronectin in serum free alpha-MEM. The cells were stained with rhodamine-phalloidin, anti-paxillin antibody (Transduction Laboratories), and Hoechst 33258 stain according to manufacturer’s instructions. The anti-paxillin antibody was detected with a FITC-conjugated anti-rabbit antibody. Fluorescence images of the cells were obtained using a deconvolution microscope and digital capture.

**ES-cell teratocarcinomas:** 2X10$^6$ wild type R1 ES cells were injected s.c. into the left hind leg of 4 Mga$5^+$ 4 Mga$5^-$ and 5 Mga$5^+$ mice. Mice were examined for palpable tumors on a weekly basis, and once tumors were detected, they were measured with calipers weekly.
CHAPTER 5: Conclusions and Future Directions
Structure-function relationships of specific glycan classes can be elucidated by targeted ablation of key enzymes in the biosynthetic pathways of glycosylation. Although glycosylation is a post-translational modification that affects many cell-surface and secreted polypeptides, and therefore its influence is pleotropic, specific biological functions have been ascribed to carbohydrate structures by studies in knockout mice. Unlike GlcNAc-TI and GlcNAc-TII, which are ubiquitously expressed, GlcNAc-TV is temporally and spatially regulated during embryogenesis and in cancer progression. The pattern of expression of GlcNAc-TV implies a possible functional specificity for β1,6GlcNAc-branched N-glycans. Indeed, the phenotypes observed in the \( Mtat5^+/\) mouse occur in cell populations expressing \( Mtat5 \) in wild type mice. GlcNAc-TV deficiency leads to a behavioral abnormality, T cell hypersensitivity and changes in leukocyte migration and adhesion. In addition, as predicted from studies with somatic tumor cell mutants with reduced GlcNAc-TV activity and β1,6GlcNAc-branched N-glycans, \( Mtat5^+/\) mice develop mammary tumors with greater latency, slower growth and show >20 fold reduction in metastasis. Our results suggest that GlcNAc-TV may regulate processes common to many cell types, such as cell motility and adhesion to ECM and receptor aggregation and related signaling.

**GlcNAc-TV deficiency affects maternal behavior:** GlcNAc-TV deficiency does not result in obvious developmental defects, as \( Mtat5^+/\) mice are viable and fertile. However, 129/sv GlcNAc-TV deficient females fail to nurse their young. This phenotype is most likely due to a behavioral defect. The behavior is strain dependent, suggesting that GlcNAc-TV may be a modifier of some other gene product, such as a signaling receptor. 129/sv females exhibit poorer maternal skills than CD1 or C57/BL-6 females and therefore GlcNAc-TV deficiency may enhance an existing phenotype. Since the
aberrant maternal behavior disappears in mixed 129/sv-CD1 or 129/sv-C57/BL-6 females, it may be possible to identify the gene interacting with \textit{Mgat5} to cause the aberrant nurturing behavior.

\textbf{GlcNAc-TV in immunity:} The data presented in Chapter 3 supports a role for GlcNAc-TV activity in the regulation of T cell proliferation and in leukocyte migration. We have shown that GlcNAc-TV deficiency results in hyperproliferation of T cells and in decreased migration of leukocytes in response to stimuli such as thioglycollate and arachidonic acid. Application of irritants causing either a T-cell dependent DTH or an immediate-type hypersensitivity resulted in exaggerated inflammatory responses in GlcNAc-TV deficient mice. In both models, the recovery time was longer in the GlcNAc-TV deficient mice, suggesting that the inflammatory responses were not dampened as efficiently as in wild type mice. We have also demonstrated activation-dependent upregulation of \textit{Mgat5} and L-PHA reactive structures in T cells. Together these data suggest that \(\beta_{1,6}\)GlcNAc-branched N-glycans may function to dampen the T cell response following antigen-mediated activation of the cells.

Inflammatory responses are terminated by the apoptosis of the immune cells. Overexpression of GlcNAc-TV in MvLu1 cells resulted in increased apoptosis, and therefore it is possible that the upregulation of GlcNAc-TV activity in activated T cells is necessary for the efficient apoptosis of these cells. \textit{Mgat5}\textsuperscript{−/−} T cells may be deficient in their ability to apoptose after antigen-dependent stimulation, which may result in the increased severity of the inflammatory response observed in the GlcNAc-TV deficient mice. In this manner, the function of GlcNAc-TV activity may be similar to the function of CTLA-4, which is expressed after the activation of T cells (Lee et al., 1998). CTLA-4 has a higher affinity for the B7 molecules, which are the stimulatory ligands for CD28. Thus, once CTLA-4 is activated, it downregulates T cell responses by outcompeting
CD28 for the B7 ligands, while physically associating with the TCR and directly inhibiting TCR signaling. Lack of CD28 signaling results in failure to produce IL-2, which protects T cells from apoptosis. CTLA-4 deficient mice develop severe autoimmunity and die 2-3 weeks after birth (Khattri et al., 1999). Although the phenotype of GlcNAc-TV deficient mice is much milder, it is possible that these mice could develop an autoimmune condition if stressed appropriately. Induced T cell dependent multiple sclerosis is a model of autoimmunity now being explored in the lab.

The ability of *Mgat5*<sup>-/-</sup> mice to clear viral or parasitic infection might also be different than in wild type mice. We are currently exploring different models of infection. The Friend leukemia virus is a well-established model for studying genetic resistance to an immunosuppressive murine virus (Hasenkrug and Chesebro, 1997), and Schistosomes infection has been studied extensively as a model of an immunosuppressive parasitic infection. Cytotoxic T cells, T helper cells, Th1 and Th2 responses, as well as antibody responses are required for clearance of infection, varying in importance depending on the pathogen. Therefore, several models will be used to study these aspects of immunity in the GlcNAc-TV deficient mouse.

Preliminary studies in the GlcNAc-TV deficient mice suggest that these mice may produce aberrant cytokine levels when challenged, or that their leukocytes may respond aberrantly to cytokines. Thioglycollate, an inflammatory agent, failed to produce a significant neutrophil and macrophage infiltration when injected into the peritoneum of *Mgat5*<sup>-/-</sup> mice. Thioglycollate stimulates the synthesis of tumor necrosis factor α (TNF-α) by macrophages and inhibits arachidonic acid production and metabolism. Therefore, our experiments suggest that while the GlcNAc-TV deficient mice may be hypersensitive to arachidonic acid metabolites, the initial delay in leukocyte migration may inhibit the production of other inflammatory cytokines, such as TNF-α, resulting in a mild and
aborted response to thioglycollate injection. This hypothesis is supported by a preliminary study of hepatic injury induced by injection of carbon tetrachloride (CCl₄), in which GlcNAc-TV deficient mice survived better than wild type littermates. Previous studies implicate interleukin-6 (IL-6), IL-10 and TNF-α in hepatic inflammation induced by CCl₄. IL-10 deficient mice displayed elevated levels of TNF-α, TGF-β1 and IL-6, resulting in an acute inflammatory burst. Slowed migration of leukocytes into the liver in GlcNAc-TV deficient mice would inhibit the acute inflammatory burst that causes mortality in this model. However, the difference in the response of GlcNAc-TV deficient mice to CCl₄ could also be due to the levels of one or several of these cytokines, their activity, or the activity of their receptors being aberrant in these mice.

**GlcNAc-TV deficiency causes delayed tumor progression and inhibits metastasis:** We crossed GlcNAc-TV deficient mice with MMTV-PyMT mice to study metastatic breast cancer. PyMT interacts with Src, Shc and PI3K. Src and Shc both act in the Ras pathway, which has been previously shown to activate Mga5. Our results show that GlcNAc-TV activity is necessary for efficient tumor progression and for metastasis. Tumor growth was retarded in Mga5⁻/⁻ mice, and metastasis was inhibited by >20 fold. Infrequent Mga5⁻/⁻ tumors exhibited altered growth rates 3-4 weeks after they became palpable to resemble wild type growth, suggesting that a second mutation occurred in these tumors. Western analysis of primary T cells and embryonic fibroblasts shows that PKB phosphorylation, an indicator of PI3K activity, is reduced in Mga5⁻/⁻ cells. Preliminary results from Western analysis of PyMT tumors suggest that Mga5⁻/⁻ null tumors also have reduced phosphorylation of PKB compared to Mga5⁺/⁺ tumors. However, PKB phosphorylation levels in fast-growing Mga5⁻/⁻ tumors resemble phosphorylation levels of Mga5⁺/⁺ tumors. Thus, it is likely that GlcNAc-TV deficiency
results in perturbation of the PI3K/PKB signaling pathway, which may be counterbalanced by a mutation in the fast-growing tumors.

The fast-growing tumors did not cause an increase in the number of metastases, suggesting that tumor growth and metastatic potential may be separate and distinct phenotypic characteristics of the $Mgat5^{-/-}$ mice. Therefore, it is possible that even though signaling through both the Ras and the PI3K pathways is similar to wild type in these tumors, lack of $\beta 1,6$GlcNAc-branched N-glycans still impedes integrin mediated focal adhesion turnover. The specificity of the defect is underscored by the efficient turnover of actin in activated $Mgat5^{-/-}$ T cells, which is directed by TCR and CD28 engagement and not by integrin engagement. The hypothesis that integrin signaling is impaired in $Mgat5^{-/-}$ fibroblasts and in PyMT$Mgat5^{-/-}$ tumor cells primarily through the Rho family of GTPases, could be tested directly by rescue experiments in which the activated Cdc42, Rac and Rho constructs would be transfected into these cells.

We have shown that $\beta 1,6$GlcNAc-branched N-glycans have a function essential for efficient metastasis, possibly independent from receptor signaling modification. This function may relate to physically aiding in focal adhesion turnover, or to extravasation and organ colonization that may be mediated by interactions with lectins. Candidates for binding partners of $\beta 1,6$GlcNAc-branched N-glycans could be the widely expressed galectins, which bind N-acetyllactosamine and have been implicated in tumor cell adhesion during metastatic spread. If the polylactosamine content of $Mgat5^{-/-}$ tumor cells is reduced, it would mean less ligand for the galectins, and therefore less efficient colonization by metastatic cells. Structural analysis of N- and O-glycans is required to determine how the $Mgat5^{-/-}$ mutation affects the distribution of residual structures.

$Mgat5$ expression may require signaling through both the Ras and the PI3K pathways and may be necessary for proper integrin signaling: The expression of lacZ in null
tumors suggests that the activation of the Ras pathway alone may not be sufficient to turn on expression from the \textit{Mgat5} locus. Preliminary results show that the level of MAPK phosphorylation in PyMT\textit{Mgat5}^{+/+} is similar to that of PyMT\textit{Mgat5}^{+/−} and PyMT\textit{Mgat5}^{++} tumors. However, expression of \textit{lacZ} is downregulated in comparison to expression in the \textit{Mgat5}^{+/−} tumors. In the rare fast growing PyMT\textit{Mgat5}^{+/−} tumors, PKB phosphorylation, as well as \textit{lacZ} expression is comparable to that observed in PyMT\textit{Mgat5}^{+/−} and PyMT\textit{Mgat5}^{++} tumors. Therefore, these results suggest that \textit{Mgat5} expression is regulated by both the Ras and the PI3K signaling pathways. This scenario is similar to the one presented in a recent report showing that the transcription factor AFX (a member of the forkhead family) requires direct phosphorylation by both PKB, a PI3K effector, and by the Ral GTPase, an effector of Ras signaling (Kops et al., 1999).

The results presented in this thesis suggest the following working model (Figure 1). Activation of receptor tyrosine kinases initiates signaling through the Ras and PI3K pathways, which results in upregulation of \textit{Mgat5} gene expression and increased β1,6GlcNAc-branched N-glycan biosynthesis. Thus, the β1,6GlcNAc-branched N-glycans on adhesion and cytokine receptors increase. Signaling initiated by integrin engagement is enhanced by β1,6GlcNAc-branching of N-glycans due to their effect of promoting receptor cluster turnover. Integrin engagement also results in the activation of Ras and PI3K pathways, leading to cytoskeletal reorganization, proliferation and migration. Further signaling through the Ras pathway, and the PI3K, amplify \textit{Mgat5} transcription and β1,6GlcNAc-branched N-glycan synthesis, creating positive feedback. In the absence of \textit{Mgat5}, integrin signaling is compromised, as indicated by PKB hypophosphorylation and reduced microfilament and focal adhesion turnover. In addition, we have shown that the positive feedback is disrupted in PyMT\textit{Mgat5}^{+/−} tumor cells. PyMT\textit{Mgat5}^{−/−} tumor cells have similar level of MAPK phosphorylation to wild
type PyMT tumor cells, indicating that the Ras pathway is intact in these cells. However, PyMT*Mgat5<sup>−/−</sup>* tumors have reduced lacZ expression from the *Mgat5* locus, a result that is not consistent with *Mgat5* expression being dependent on the Ras pathway alone. The dependence of *Mgat5* expression on PI3K signaling is further illustrated by the enhanced lacZ expression in fast growing PyMT*Mgat5<sup>−/−</sup>* tumors, where PKB phosphorylation are restored to wild type levels.

Recently, several papers have highlighted the importance of integrin signaling in T cell activation. Signaling mediated by LFA-1, the lymphocyte adhesion receptor for ICAM-1, was shown to be necessary for cytoskeletal rearrangement and TCR internalization (Grakoui et al., 1999; Bachmann et al., 1997). In our experiments, aberrant cytoskeletal rearrangement was observed in *Mgat5<sup>−/−</sup>* T cells, fibroblasts and PyMT tumor cells. It is therefore possible that the glycoproteins affected most critically by β1,6GlcNAc-branched N-glycosylation are the integrins, and that the phenotypes observed in the *Mgat5<sup>−/−</sup>* mouse are due to a common defect, the aberrant function of integrins.

*The biological significance of GlcNAc-TV activity may be investigated in worm:* As demonstrated by this work and by work with other glycosyltransferase-deficient mice, some glycosylation effects are cell nonautonomous and therefore must be studied in a whole organism. Although a great deal of information can be obtained from studies with mice, these mammals are very complex. Enhancer/suppressor screens to identify gene products modified by glycosylation are practically difficult and expensive in mice. *C. elegans* provides a genetically tractable and simpler alternative. The components of the Ras and the PI3K signaling pathways are conserved in *C. elegans*, as is GlcNAc-TV. Mutations in the *let* genes (the worm Ras orthologues) lead to migration defects in the sex myoblast cells. Mutations in the PI3K orthologue (*age1*) result in altered longevity.
Therefore, screens for enhancer/suppressor mutations on gly-2 (the worm GlcNAc-TV gene) or other glycosyltransferase deficient worms would be useful in identifying key substrates of glycosylation, which may aid in further understanding the molecular basis for the mammalian phenotype.
Figure 1  Schematic of hypothesis for GlcNAc-TV function in cell adhesion.

A) Signaling through receptor tyrosine kinases initiates signaling through the Ras and PI3K pathways, which result in upregulation of *Mgat5* expression and increased β1,6GlcNAc-branched N-glycans. Thus, the β1,6GlcNAc-branched N-glycans on adhesion and cytokine receptors are increased. Signaling initiated by α5β1 integrin engagement is enhanced by β1,6GlcNAc-branched N-glycans and results in the activation of FAK-Src and of PI3K. The activation of these pathways leads to cytoskeletal reorganization, proliferation, migration and enhancement of signaling through the Ras and the PI3K pathways, which in turn amplifies *Mgat5* transcription and β1,6GlcNAc-branched N-glycan synthesis, creating positive feedback. B) In the absence of *Mgat5*, signaling initiated by integrin adhesion to ECM is compromised, possibly by physically inhibiting focal adhesion formation and turnover. Integrin Signaling through the PI3K is affected in embryonic fibroblasts and in PyMT*Mgat5* tumor cells, and the cytoskeletal organization of PyMT*Mgat5* tumor cells indicates that signaling through Cdc42, positioned downstream of integrins and upstream of PI3K is compromised. The disruption of positive feedback in GlcNAc-TV deficient cells is evident by the reduced expression of lacZ from the *Mgat5* locus in the PyMT*Mgat5* tumors, where PKB is hypophosphorylated.
Integrins → Cdc42 → PI3 kinase → PKB

Cytoskeletal reorganization

FAK + Src → Rac → 3PPIs

N-glycans?

Cytokine Receptor tyrosine kinases

Grb-2

Shc + Sos

Ras

Raf-1

MAP kinase

Jun + Fos

β1,6GlcNAc-branched N-glycan synthesis

Mgat5

Proliferation Motility

Ets
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