Isolation, characterization and inactivation of the mouse \textit{Mgat3} gene: the bisecting \textit{N}-acetylglucosamine in asparagine-linked oligosaccharides appears dispensable for viability and reproduction

John J. Priatel, Mohan Sarkar\(^1\), Harry Schachter\(^1\) and Jamey D. Marth\(^2\)

Howard Hughes Medical Institute, Department of Medicine, and the Division of Cellular and Molecular Medicine, 9500 Gilman Drive 0625, CMM-W 321-B, University of California at San Diego, La Jolla, CA 92093, USA and the \(^1\)Department of Biochemistry, The Hospital for Sick Children and the University of Toronto, 555 University Avenue, Toronto, Ontario, MSG 1X8, Canada

\(^2\)To whom correspondence should be addressed: Howard Hughes Medical Institute, 9500 Gilman Drive 0625, University of California at San Diego, La Jolla, CA 92093, USA

The biosynthesis of complex asparagine (\textit{N})-linked oligosaccharides in vertebrates proceeds with the linkage of \textit{N}-acetylglucosamine (GlcNAc) to the core mannose residues. UDP-\textit{N}-acetylglucosamine:\textit{β}-d-mannoside \(\beta\)-1-4 \textit{N}-acetylglucosaminyltransferase III (GlcNAc-TIII, EC2.4.1.144) catalyzes the addition of GlcNAc to the mannose that is itself \(\beta\)-1-4 linked to underlying \textit{N}-acetylglucosamine. GlcNAc-TIII thereby produces what is known as a 'bisecting' GlcNAc linkage which is found on various hybrid and complex N-glycans. GlcNAc-TIII can also play a regulatory role in \textit{N}-glycan biosynthesis as addition of the bisecting GlcNAc eliminates the potential for \(α\)-mannosidase-II, GlcNAc-TIII, GlcNAc-TIV, GlcNAc-TV, and core \(α\)-1-6-fucosyltransferase to act subsequently. To investigate the physiologic relevance of GlcNAc-TIII function and bisected N-glycans, the mouse gene encoding GlcNAc-TIII (\textit{Mgat3}) was cloned, characterized, and inactivated using Cre/loxP site-directed recombination. The \textit{Mgat3} gene is highly conserved in comparison to the rat and human homologs and is normally expressed at high levels in mammalian brain and kidney tissues. Using fluorescence \textit{in situ} hybridization (FISH), the \textit{Mgat3} gene was regionally mapped to chromosome 15E11, near the Scn8a sodium channel gene at 15F1. Following homologous recombination in embryonic stem cells and Cre mediated gene deletion, \textit{Mgat3}-deficient mice were produced that lacked GlcNAc-TIII activity and were deficient in E\(_4\)-PHA visualized GlcNAc-bisected N-linked oligosaccharides. Nevertheless, GlcNAc-TIII deficient mice were found to be viable and reproduced normally. Moreover, such mice exhibited normal cellularity and morphology among organs including brain and kidney. No alterations were apparent in circulating leukocytes, erythrocytes or in serum metabolite levels that reflect kidney function. We thus find that GlcNAc-TIII and the bisecting GlcNAc in N-glycans appear dispensable for normal development, homeostasis and reproduction in the mouse.

\textit{Key words:} GlcNAc/brain/kidney/biosynthesis

\section*{Introduction}

An early step in complex and hybrid \textit{N}-glycan biosynthesis can be initiated by GlcNAc-TIII which adds a GlcNAc monosaccharide in \(β\)-1-4 linkage to the \(β\)-1-4 linked mannose of the processed mannose core (reviewed in Schachter \textit{et al.}, 1983). Although the function of the resulting 'bisecting' GlcNAc is not well understood, this modification can inhibit completely the action of other enzymes in subsequent \textit{N}-glycan biosynthesis (\(α\)-mannosidase-II, GlcNAc-TIII, GlcNAc-TV, core \(α\)-1-6-fucosyltransferase) and inhibit partially UDP-Gal:GlcNAc \(β\)-1-4 galactosyltransferase (Schachter, 1986), suggesting a regulatory role in the formation of complex and hybrid \textit{N}-glycans. While bisected \textit{N}-linked oligosaccharides have not thus far been shown to specifically interact with endogenous lectin receptors, altered exogenous lectin binding has been reported (Cummings and Kornfeld, 1982; Narasimhan \textit{et al.}, 1986) as well as decreased accessibility of cell surface Gal residues to Gal-binding lectins in a Chinese hamster ovary (CHO) glycosylation variant line expressing a dominant mutation in GlcNAc-TIII (Campbell and Stanley, 1984; Stanley \textit{et al.}, 1991).

GlcNAc-TIII activity was first described in the hen oviduct (Narasimhan, 1982) and has subsequently been found in various systems including the rat liver during hepatocarcinogenesis (Narasimhan \textit{et al.}, 1988b; Nishikawa \textit{et al.}, 1988a; Pascale, 1989), rat kidney (Nishikawa \textit{et al.}, 1988b), rat brain (Nishikawa \textit{et al.}, 1988b), human B lymphocytes (Narasimhan \textit{et al.}, 1988a), HL60 cells (Koenderman \textit{et al.}, 1987), Novikoff ascites tumor cells (Koenderman \textit{et al.}, 1989), and CaCO-2 cells (Brockhausen \textit{et al.}, 1991). The gene encoding GlcNAc-TIII has been isolated from vertebrate sources including the rat, human (MGAT3), and mouse (\textit{Mgat3}) (Nishikawa \textit{et al.}, 1992; Ihara \textit{et al.}, 1993; Bhaumik \textit{et al.}, 1995). Expression of \textit{Mgat3} RNA appears high in mouse brain and kidney tissue and increased expression following gene transfer in various cell lines has been reported to suppress cellular susceptibility to NK cell cytotoxic mechanisms; promote spleen colonization, suppress lung metastatic activity of the B16 mouse melanoma via increased E-cadherin binding; and suppress expression of hepatitis B virus (Miyoshi \textit{et al.}, 1995; Yoshimura \textit{et al.}, 1995a, 1996a,b). In addition, high levels of GlcNAc-TIII activity and bisecting N-glycans have been reported in cells derived from patients with chronic myelogenous leukemia in blast crisis (Yoshimura \textit{et al.}, 1995b,c). N-glycans associated with metastasis frequently involve altered branching at the trimannosyl core, by GlcNAc-TV mediated \(β\)-1-6 GlcNAc addition (Dennis \textit{et al.}, 1987; Yousefi \textit{et al.}, 1991; Saitoh \textit{et al.}, 1992). As these enzymes can compete for common substrates, a functional interplay in oncogenesis between GlcNAc-TIII and GlcNAc-TV
appears possible and may be revealed by modifying oligosaccharide production in the context of tumorigenic physiology.

At present it is clear that complex N-linked oligosaccharide biosynthesis is required in embryonic development (Ioffe and Stanley, 1994; Metzler et al., 1994) although the minimal structure necessary for normal ontology is not yet defined. This regulated biosynthetic pathway often generates oligosaccharide structures bearing multiple antennae, suggesting the possibility that unique biological information is contained along each branch, information that may be perceived following the ablation of specific branching/diversification steps as controlled by specific glycosyltransferases and glycosidases. It is also possible that complex N-glycans may function in part through multivalent interactions as a result of multiantennary structures harboring identically modified branches or termini.

In continuing to investigate these possibilities, we have created mice that lack a functional MgaT3 gene, are devoid of GlcNAc-TIII activity, and deficient in the bisecting GlcNAc using Cre-loxP site-directed mutagenesis (reviewed in Marth, 1994, 1996; Hennet et al., 1995).

Results

Cloning and characterization of the mouse MgaT3 gene

Using polymerase chain reaction (PCR) and rat genomic DNA as template, the GlcNAc-TIII coding sequence (Nishikawa et al., 1992) was isolated for use as a probe in cloning of the mouse MgaT3 gene. Mouse genomic DNA encoding GlcNAc-TIII in germline configuration was subsequently obtained from a 129/SvJ genomic DNA library in lambda phage by cross-hybridization with the PCR-derived rat probe. Restriction enzyme mapping and sequence analyses on both DNA strands revealed the presence of an uninterrupted GlcNAc-TIII coding sequence predicted to contain 538 amino acids since translation by eukaryotic ribosomes would be expected to initiate virtually 100% of the time at the first 5' AUG in sequence context... ANNAUGN... (Kozak, 1991). GlcNAc-TIII forms lacking the N-terminal methionine and lysine displayed in Figure 1 may occur in the rat which exhibits a pyrimidine residue at nucleotide -3 from this 5' AUG (Nishikawa et al., 1992). Unexpectedly, additional comparisons between the MgaT3 gene isolated herein and that published (Bhaumik et al., 1995) revealed 21 nucleotide differences that generated seven amino acid changes between these two mouse sequences (Figure 1). Curiously, in all but three instances (positions 159, 243, and 536), the amino acid differences between the two mouse sequences nevertheless maintain identity with either the rat or human GlcNAc-TIII sequence. Both mouse MgaT3 sequences were independently obtained from a 129 genomic library, although 129 strain variations of DNA used may explain the differences between the two predicted mouse GlcNAc-TIII sequences (see Discussion).

![Alignment of mouse, rat, and human GlcNAc-TIII amino acid sequences.](attachment:image.png)

Fig. 1. Comparison of putative mouse, rat, and human GlcNAc-TIII sequences. Identities between mouse and rat or human amino acids are denoted (dots). Gaps are present in the human sequence (hyphens). Differences between the mouse GlcNAc-TIII sequence obtained and that published (Bhaumik et al., 1995) are indicated as amino acid changes or silent substitutions (*) above the mouse sequence reported herein. The nucleotide sequence has been deposited to Genbank, accession number U66844.
With the mouse genomic Mgat3 probe, fluorescence in situ hybridization (FISH) to normal mouse lymphocyte chromosomes was undertaken. Regional assignment of Mgat3 to mouse chromosome 15 at position E11 was determined following analyses of 20 well-spread metaphases as represented in Figure 2A. Positive hybridization signals were noted in all 20 metaphases and were visualized on both homologs in 85% of these metaphase spreads (17/20). Although the signal was distinctly visualized, some background hybridization was observed, perhaps reflecting repetitive sequence present within the 16 kilobase (kb) genomic Mgat3 probe. Chromosome assignment was initially determined by the banding karyotype (see Materials and methods) and confirmed by a chromosome 15-specific probe med/Scn8a that maps to 15Fl (Figure 2B; Burgess et al., 1995; Kohrman et al., 1995). Our results are in agreement with previous reports that assigned Mgat3 to mouse chromosome 15 (Bhaumik et al., 1995) and in a region likely syntenic with the human Mgat3 homologue positioned at chromosome 22q13.1 (Ihara et al., 1993).

Expression of Mgat3 RNA among various normal mouse tissues was determined using a 1.8 kb Mgat3 probe containing the entire mouse GlcNAc-TIII coding sequence. Results indicated that highest steady-state RNA expression levels occur in brain and kidney, followed by colon, small intestine, lung, thymus, stomach, and ovary (Figure 3). This expression pattern is similar to results obtained in other studies among various tissue samples (Nishikawa et al., 1992; Bhaumik et al., 1995).

Generation of mice harboring a null allele at the Mgat3 locus

To generate a null genotype in mice, the genomic Mgat3 isolate was used to produce a gene targeting vector by cloning genomic fragments into the pflox plasmid (Figure 4A). Following homologous recombination in embryonic stem (ES) cells, the GlcNAc-TIII protein-encoding exon was flanked by loxP sites, as were the adjacent selectable markers herpes simplex virus thymidine kinase (Tk) and neomycin phosphotransferase (Neo). The resulting targeted Mgat3 allele (Figure 4B, Mgat3Flkane) was then a substrate for Cre recombination activity in producing two types of altered Mgat3 alleles. Recombination between distal loxP sites generated a null allele (Mgat3A) by deletion of all intervening DNA, while recombination between loxP sites flanking the TK and Neo cistrons generated a conditional mutation (Mgat3B) with loxP sites flanking the GlcNAc-TIII protein encoding sequence (Figure 4C). This presumptive conditional mutation achieved in ES cells allows the production of mice bearing a functional Mgat3 allele that can be deleted by transgenic Cre recombination expression should inactivation of both Mgat3 alleles lead to multisystemic phenotypes and early developmental lethality in mice (reviewed in Marth, 1994, 1996).

Homologous recombination was detected by PCR at a frequency of 1/38 G418-resistant clones (10 positives of 384 clones screened). Southern blotting of eight PCR-positive clones confirmed that in each case one Mgat3 allele exhibited the expected structural alteration (Mgat3Flkane) in comparison to germline 129 DNA (Figure 5A and data not shown). Using a loxP probe, five of eight clones examined did not contain the 3' loxP site (Figure 5B), likely the result of homologous recombination within the loxP flanked genomic Mgat3 sequence.

To produce ES cell subclones that contained the Type I (systemic) and Type II (conditional) Mgat3 mutations, ES clone 1H8 was subjected to transient Cre expression by electroporation of pMC-Cre and subsequent selection in the presence of ganciclovir. Subclones resistant to gancyclovir were isolated and analyzed by genomic Southern blotting. Using a loxP or genomic probe, 16 of 18 gancyclovir resistant clones screened had undergone a Type I recombination and deletion while the remaining two exhibited the Type II deletion (Figure 5C,D and data not shown). Parental ES cells and 1H8 subclones 1 and 2 were analyzed for GlcNAc-TIII activity in vitro. Comparison of GlcNAc-TIII enzyme activities among extracts of R1 ES cells (Mgat3WTWT), Type I clones (Mgat3WT/A), and Type II clones (Mgat3WT/P) yielded 100%, 37%, and 97%, respectively (data not shown).

Chimeric mice were initially generated from Type I (Mgat3WT/Mgat3A) ES cell 1H8 subclone #2 to determine whether systemic loss of Mgat3 function would produce phenotypic results indicating a developmental role for the bisecting GlcNAc in N-glycans. Offspring heterozygous for the Mgat3A allele were produced by crossing chimeric mice with inbred C57BL/6 mates. These heterozygotes were normal and were crossed to generate embryos and mice that were homozygous for the Mgat3A allele. Genotypes were subsequently determined by tail samples of offspring produced from parents heterozygous for the Mgat3A allele. With a genomic Mgat3 probe and NdeI-digested DNA, the presence of all three genotypes was observed (Figure 6A). An NdeI restriction fragment length polymorphism was found between 129 and C57BL/6 mouse strains, thus producing an Mgat3WTBL6-derived fragment of approximately 4 kilobases (kb) in comparison to the 8.5 kb fragment observed in wild-type 129 DNA (compare with results in Figure 5A). Using the entire GlcNAc-TIII coding region as a probe, Cre recombination in ES cells was found to have resulted in both excision and degradation of Mgat3 DNA flanked by loxP sites as no hybridization signal was found in genomic DNA samples from Mgat3A/Mgat3A mice (Figure 6B).

Analyses of Mgat3-null mice

Results from multiple matings indicated that homozygous deletion of the Mgat3 gene was not lethal in embryonic development (Table I). Moreover, homozygous Mgat3A/Mgat3A mice appeared normal (see further below) and were subsequently found to be fertile in crosses with either inbred C57BL/6 or Mgat3A/Mgat3A mice. From crosses performed, offspring were generated of both sexes and bearing Mgat3 genotypes at frequencies representing a normal Mendelian distribution (Table I).

To confirm that the Mgat3A allele resulted in loss of GlcNAc-TIII enzyme activity, a synthetic substrate specific for GlcNAc-TIII was used in vitro to measure enzyme activity in tissue extracts from mice bearing Mgat3WT or Mgat3A alleles (Khan et al., 1994, and see Materials and methods). With this substrate, GlcNAc-TIII activity levels were found to be reduced by 50% in heterozygous extracts of brain and kidney (Figure 7), tissues with highest GlcNAc-TIII levels normally, and loss of all measurable GlcNAc-TIII activity (less than 0.02 nmol/mg protein per h) was also noted in extracts of brain, kidney, thymus, liver, and spleen derived from Mgat3A/Mgat3A mice (Figure 7 and data not shown). These results confirm that the mutation generated in the Mgat3 allele (Mgat3A) is a null mutation as would be expected following the deletion of the entire GlcNAc-TIII coding sequence.

Loss of Mgat3-encoded GlcNAc-TIII activity would be expected to result in loss of bisecting GlcNAc residues in
Fig. 2. Regional chromosomal localization of the \textit{Mgat3} gene by fluorescence in situ hybridization. (A) A representative metaphase preparation is shown in which \textit{Mgat3} probe hybridization was detected on both homologues of chromosome 15 as described in Materials and methods. (B) Double color FISH showing DIG-labeled \textit{Mgat3} detected with FITC-avidin (yellow, as in A) and biotinylated chromosome 15 marker probe (med/Scn8a) detected with rhodamine-anti-DIG (red) as described in Materials and methods. \textit{Mgat3} is localized adjacent and telomeric to med/Scn8a.
Isolation, characterization, and inactivation of the mouse \textit{Mgat3} gene

Fig. 3. Expression of \textit{Mgat3} RNA among normal mouse tissues. Five micrograms of total cellular RNA was analyzed from each tissue sample. A 1.8 kilobase \textit{Bam} III \textit{Mgat3} genomic fragment containing the entire GlcNAc-Tlll protein-encoding region was used as a probe. The bottom panel represents the ethidium-stained profile of RNA levels analyzed.

\textbf{Discussion}

The biosynthetic pathway leading to complex and hybrid N-glycan production is conserved in mammalian organisms and is necessary for embryonic development (Ioffe and Stanley, 1994; Metzler \textit{et al}., 1994). Additional experiments are needed to define the N-linked oligosaccharide structures required for mouse postembryonic day 9 ontogeny. GlcNAc-Tlll was a candidate enzyme providing for this function as it can act

N-glycans, a prediction that can be addressed using the lectin \textit{E}_{4}-PHA which has been reported to bind specifically to bisected N-glycans when employed under certain experimental conditions (Kobata and Yamashita, 1989, 1993; Miyoshi \textit{et al}., 1995; Yoshimura \textit{et al}., 1995a,c, 1996b). Using extracts from kidneys harboring the three \textit{Mgat3} genotypes (\textit{Mgat3}^{WT}/\textit{Mgat3}^{WT}, \textit{Mgat3}^{WT}/\textit{Mgat3}^{A}, \textit{Mgat3}^{A}/\textit{Mgat3}^{A}), a deficiency in \textit{E}_{4}-PHA binding was observed to correlate with the presence of the null genotype (Figure 8). \textit{L}-PHA binding was also employed and was not found to be greatly affected, although perhaps a slight increase may occur in extracts bearing \textit{Mgat3}-null allelic structure (Figure 8, lower panel). Background bands obtained using both lectins represent endogenous biotin and may also include, in the case of \textit{E}_{4}-PHA, reactivity not specific for bisecting GlcNAc residues. Nevertheless, these results support the view that the \textit{Mgat3} allele is responsible for encoding GlcNAc-Tlll activity necessary for the production of bisecting GlcNAc residues in N-glycans.

While mice lacking a functional \textit{Mgat3} allele did not display overt phenotypic consequences, additional studies were undertaken to determine whether some tissues and physiologic systems known to express GlcNAc-Tlll remained nominal. Brain and kidney tissues which normally exhibit relatively high levels of GlcNAc-Tlll expression were analyzed histologically to detect morphologic alterations in the absence of GlcNAc-Tlll activity. From these studies there did not appear to be any change in cellularity or morphogenic organization of these tissues (data not shown). Furthermore, behavioral or neuromuscular abnormalities in \textit{Mgat3}-null mice have not been observed, with the oldest of such animals presently reaching the age of 12 months. We also analyzed the steady-state peripheral hematopoietic compartment in \textit{Mgat3-null mice.} Results indicated a normal profile, size, and morphology of circulating hematopoietic cells (Table I). Analyses of serum metabolites reflecting kidney function also revealed normal profiles in \textit{Mgat3-null samples} (Table I). Therefore, in a relatively stress-free laboratory environment, mice lacking a functional \textit{Mgat3} gene develop, function, and reproduce normally.
A

Genomic Clone 129/SvJ

pflox Vector

B

Mgat3

Targeting Vector

Mgat3 WT-129

Mgat3 F[tkneo]

C

Mgat3Δ

Mgat3F

Mgat3

+Cre
+Ganciclovir

Type 1 Deletion

Type 2 Deletion
Isolation, characterization, and inactivation of the mouse \textit{Mgat3} gene

Fig. 5. Homologous and Cre-generated recombination at the \textit{Mgat3} locus in embryonic stem (ES) cells. (A) The presence of a targeted \textit{Mgat3} allele is observed by Southern blot analysis of seven G418-resistant PCR-positive ES cell clones, as compared to wild-type 129 DNA. (B) Use of a \textit{loxP}-specific probe indicated that two of seven \textit{Mgat3}-targeted clones retained all three \textit{loxP} sites. (C) Using the 1H8 \textit{Mgat3}-targeted ES clone, Cre recombination and ganciclovir selection resulted in two types of recombination events (see Figure 4C) with subclone 1H8-1 exhibiting a Type II recombination, while subclones 1H8-2-4 underwent Type I deletions. Analysis of the Type II recombination (clone 1H8-1) revealed a \textit{loxP}-hybridizing \textit{Mgat3} allele that is expectedly similar in size to the wild-type 129-derived allele (D), the latter of which lacks \textit{loxP} sites (compare with C). Five micrograms of ES cell DNA was analyzed in the above studies.

relatively early in the biosynthesis of complex and hybrid N-linked oligosaccharides, immediately after the addition of a GlcNAc residue to the Manol-3 arm of the core by GlcNAc-TI. The resulting bisecting GlcNAc residue prevents subsequent action by \(\alpha\)-mannosidase II, GlcNAc-TII, GlcNAc-TIV, GlcNAc-TV, and core \(\alpha\)-1-fucosyltransferase, thereby limiting N-linked oligosaccharide biosynthesis to bisected hybrid forms bearing a single Manol-3-linked antenna which may be extended in various ways (Schachter \textit{et al.}, 1983; Schachter, 1986). Therefore, loss of GlcNAc-TIII activity and lack of bisecting GlcNAc residues may also result in additional N-glycan branching with multiple antenna formation and decreased abundance of hybrid structures in glycoproteins. Regardless of the outcome, we reasoned that a mouse lacking GlcNAc-TIII activity would be useful in further determining the physiologic roles of complex and hybrid N-linked oligosaccharides, and may ultimately be a necessary reagent in further studies to define N-glycan structure and function, as well as any functional redundancy among N-linked oligosaccharide structures.

The \textit{Mgat3} gene encodes GlcNAc-TIII and is highly conserved among vertebrates thus far studied. A single allelic copy
exists in the mammalian genome and the predicted amino acid sequence of mouse GlcNAc-TII is over 90% identical to homologues analyzed in rat and human DNA (Nishikawa et al., 1992; Ihara et al., 1993) and over 98% identical to the previously published mouse sequence (data not shown and Bhaumik et al., 1995). Although initiation of translation in the mouse may well provide two additional amino acids (M-K-) at the N-terminus (Figure 1), this addition may not occur as frequently in the rat as the first 5' AUG identified is not in a sequence context that would be recognized at high frequency by eukaryotic ribosomes (a pyrimidine is present at position -3; Kozak, 1991). Whether a similar situation exists in human Mgat3 GlcNAc-TII is not clear at present as the reported 5' untranslated sequence does not extend sufficiently into that region (Ihara et al., 1993). Unexpectedly, seven amino acid changes occur between the two mouse Mgat3 sequences thus far analyzed, while four of these changes are in fact conserved in rat or human sequences (Figure 1). Both mouse sequences were derived from 129 DNA, so these changes may reflect divergence between 129/Sv and 129/SvJ mouse strains. Previous chromosomal localization studies of mouse and human homologues (Ihara et al., 1993; Bhaumik et al., 1995) are fully consistent with the study herein, which maps the Mgat3 gene to mouse chromosome 15 at E11 in a region likely syntenic with the human genome and homolog at chromosome 22q.13.1.

The Mgat3 expression profile at the RNA level appears conserved among vertebrate cell types with high levels present in kidney of and rat (Figure 3; Nishikawa et al., 1992; Bhaumik et al., 1995). Additionally, Mgat3 expression was also found at high levels in brain, and to a lesser amount in other tissues surveyed herein and previously. Like some other glycosyltransferases cloned and characterized to date (Schachat, 1994), the putative GlcNAc-TIII amino acid sequence exists uninterrupted by introns and within an RNA transcript much larger than necessary to encode the enzyme. It is possible that 5' and 3' Mgat3 untranslated sequences may reside in other exons and may play some role in enzyme production yet to be disclosed, although regulation of Mgat3 expression and function may be completely accomplished by mechanisms involving transcriptional control, intracellular localization, and N-glycan substrate availability.

Studies herein have succeeded in generating and phenotyping mice lacking GlcNAc-TIII activity with a resulting deficiency in N-glycans harboring bisecting GlcNAc residues. We suspected that lack of GlcNAc-TIII and bisecting GlcNAc residues might promote increased branching of N-linked oligosaccharides, by GlcNAc-TV for example. However, we did not observe any significant increase in L-PHA reactivity in the kidney (Figure 8). Several explanations may be considered including the possible saturation of kidney GlcNAc-TV with other substrates, possibly low GlcNAc-TV activity levels in the normal kidney, or perhaps the obvious technical limitations in using lectin blotting techniques for analyzing whole tissue types. Additional structural analysis, as possible, will be needed to confirm that the deficiency observed reflects a total loss of bisecting GlcNAc residues. Although we cannot from our data conclude against the possibility of some remaining bisecting GlcNAc residues as a result of a GlcNAc-TIII isozyme not encoded by the Mgat3 gene, Southern genomic blotting did not reveal any Mgat3 cross-hybridizing elements, and the mutation generated herein deleted the entire protein-encoding exon with a resulting deficiency in enzyme activity and Eo-PHA lectin binding among homozygous samples; thus, we do not have sufficient reason at present to invoke the existence of a distinct gene encoding a GlcNAc-TIII isozyme.

Mice devoid of GlcNAc-TIII appeared normal and lacked any overt behavioral or neuromuscular alterations. They were similar in weight to wild-type offspring (±10%), matured equally well with control littersmates, and reproduced normally (Table I and data not shown). Additionally, tissues that normally exhibit the highest levels of GlcNAc-TIII expression, including brain and kidney, were similar in wet-weight mass in mice lacking a functional Mgat3 allele. Furthermore, no changes in cellular morphology were found when these tissues were histologically analyzed (data not shown). The identity and characteristics of circulating leukocytes and red blood cells were also normal in the absence of a functional Mgat3 gene, and serum metabolite levels used to assess kidney function were unaffected (Table I).

Although our results do not at present indicate a physiologic role for GlcNAc-TIII and bisecting GlcNAc residues in N-linked oligosaccharides, we do not view this data as evidence that a function for the bisecting GlcNAc residue does not exist. Additional experiments with Mgat3-null mice can be focused on the potential roles for GlcNAc-TIII action in accessory processes, such as cell metastasis, tumorigenesis, and homing.

Fig. 6. Heterozygous and homozygous mutations at the Mgat3 allele in intact mice. (A) Analyses of tail DNA isolated from animals generated from matings between mice heterozygous for the Mgat3<sup>a</sup> allele. The wild-type Mgat3 allele is derived from the C57BL/6 background and is smaller than the 129-derived Mgat3 allele using NdeI in Southern blot analysis, due to a restriction fragment length polymorphism between 129 and C57BL/6 strains. (B) Southern blot analyses using the Mgat3 coding sequence as a probe confirm a complete absence of hybridization in Mgat3<sup>b</sup>/Mgat3<sup>a</sup> DNA samples as a result of Cre recombination and degradation of excised DNA. Five micrograms of DNA was analyzed in the above studies.
isolation, characterization, and inactivation of the mouse Mgat3 gene

(Narasimhan et al., 1988b; Nishikawa et al., 1988a; Pascale et al., 1989; Miyoshi et al., 1995; Yoshimura et al., 1995a-c, 1996a,b). Furthermore, immunoglobulin structures which contain bisecting GlcNAc (Savvidou et al., 1984) may depend upon that modification for structural stability and functional integrity, possibilities that can be assessed in experiments that investigate B cell development and activation using various antigenic challenge regimes. Hematopoietic reconstitution and analyses of tumorigenesis in Mgat3-null mice bearing various oncogenic lesions are also relevant experiments to undertake, and can include the production of cell lines bearing either the Mgat3^{b/Mgat3} or Mgat3^{a/Mgat3} genotype—the latter allowing for deletion of the Mgat3 gene by Cre recombinase expression systems now developed. Even though a significant percentage of genetically manipulated mice bearing null mutations derived by gene-targeting techniques appear normal in the laboratory environment, the high degree of evolutionary conservation of alleles such as Mgat3 throughout mammalian radiation may well be indicative of yet to be established selective advantages for the organism by enabling appropriate responses to a changing environment.

Materials and methods

Genomic DNA isolation and analysis of the mouse Mgat3 gene. A 1.6 kilobase GlcNAc-TIII-encoding cDNA probe was amplified from 5 ng of rat genomic DNA using primers designed with the rat cDNA sequence (Nishikawa et al., 1992). The primers were as follows: 5'TCCTATGTCACTTCCGAGAGAATGCTCCCTCCCGACCCAGCCTACTACGTTCC3' and 5'C<:CCrcCGTTGTATCCAACTTGCC3'. The PCR product was purified on a 1.7% agarose gel, verified by restriction analyses and cloned into pUC19. The cloned insert was isolated, purified on a 

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\begin{array}{ccc}
\text{Parental genotypes} & \text{Mgat3}^{WT} & \text{Mgat3}^{WT} & \text{Mgat3}^{a/b} \\
\hline
\text{Mgat3}^{a/b}/\text{Mgat3}^{a/b} \times \text{Mgat3}^{a/b} & 100 & 179 & 103 \\
\text{Mgat3}^{a/b}/\text{Mgat3}^{a/b} & 45 & 64 & - \\
\text{Mgat3}^{a/b}/\text{Mgat3}^{a/b} \times \text{Mgat3}^{a/b} & 55 & 57 & - \\
\text{Mgat3}^{a/b}/\text{Mgat3}^{a/b} & - & - & 44 \\
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<tr>
<td>Red blood cells (x10^12/μl)</td>
<td>8730 ± 410</td>
<td>8780 ± 370</td>
</tr>
<tr>
<td>Hemoglobin (g/l)</td>
<td>148 ± 20</td>
<td>146 ± 39</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>45.8 ± 1.5</td>
<td>46.3 ± 2.0</td>
</tr>
<tr>
<td>Mean cell volume (fl)</td>
<td>52.5 ± 1.6</td>
<td>52.3 ± 1.4</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin (pg)</td>
<td>16.9 ± 0.8</td>
<td>167 ± 0.9</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin concentration (g/l)</td>
<td>325 ± 1.0</td>
<td>319 ± 1.1</td>
</tr>
<tr>
<td>Red cell distribution width (%)</td>
<td>16.7 ± 1.1</td>
<td>16.0 ± 1.0</td>
</tr>
<tr>
<td>Platelets (x10^12/μl)</td>
<td>882 ± 133</td>
<td>871 ± 75</td>
</tr>
<tr>
<td>Mean platelet volume (fl)</td>
<td>4.3 ± 0.2</td>
<td>4.5 ± 0.2</td>
</tr>
</tbody>
</table>

n = 10 for wild-type mice, and n = 12 for Mgat3 null mice.

<table>
<thead>
<tr>
<th>Renal panel</th>
<th>Wild-type</th>
<th>Mgat3^{a/b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicarbonate (mM)</td>
<td>14.2 ± 2.4</td>
<td>14.2 ± 2.9</td>
</tr>
<tr>
<td>Chloride (mM)</td>
<td>118.5 ± 2.3</td>
<td>118.6 ± 3.7</td>
</tr>
<tr>
<td>Sodium (mM)</td>
<td>151.0 ± 2.7</td>
<td>1503 ± 4.6</td>
</tr>
<tr>
<td>Potassium (mM)</td>
<td>5 ± 0.7</td>
<td>5.2 ± 1.0</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>9.3 ± 1.0</td>
<td>9.3 ± 1.3</td>
</tr>
<tr>
<td>Blood urea nitrogen (mM)</td>
<td>8.6 ± 1.4</td>
<td>9.0 ± 2.9</td>
</tr>
<tr>
<td>Phosphate (mM)</td>
<td>2.9 ± 0.5</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>Calcium (mM)</td>
<td>2.3 ± 0.2</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Creatinine (μM)</td>
<td>24 ± 6</td>
<td>29 ± 10</td>
</tr>
<tr>
<td>Total bilirubin (μM)</td>
<td>65 ± 18</td>
<td>82 ± 3.8</td>
</tr>
<tr>
<td>Direct bilirubin (μM)</td>
<td>3.4 ± 1.4</td>
<td>6.2 ± 3.9</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>12 ± 1</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>44 ± 2</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>ALT (alanine aminotransferase) (IU/l)</td>
<td>26 ± 5</td>
<td>27 ± 13</td>
</tr>
<tr>
<td>AST (aspartate aminotransferase) (IU/l)</td>
<td>177 ± 59</td>
<td>188 ± 75</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/l)</td>
<td>76 ± 34</td>
<td>65 ± 14</td>
</tr>
</tbody>
</table>

n = 5 for wild-type and Mgat3 null mice. Hematology and renal panel data are presented as the mean ± standard error of the mean.
 priming, approximately every 400 bases in a strategy that encompassed both DNA strands.

**DNA sequencing**

Sequencing was employed using 500 ng of template DNA plus 3.2 pmol of relevant primer in a 12 μl volume. 8 μl of ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (with AmpliTaq DNA Polymerase, FS) was added to give a final volume of 20 μl. MJ Research DNA Engine Peltier Thermal Cycler 200 was operated for the cycle sequencing. The reaction parameters included a hot start at 96°C, denaturation at 96°C for 10 s, annealing temperature for 5 s, and elongation at 60°C for 4 min, repeated for 25 cycles, followed by cooling to 4°C. Dye nucleotide cleanup was accomplished by spin column purification using MicroSpin G50 Columns from Pharmacia. Gels were run on an ABI 373A autosampler under the following conditions: 6% acrylamide, 8.3 M urea, 1 × TBE gel, 1 × TBE as buffer system, 24 cm plates, 30 W, 12 h. Before loading, samples are given 3–4 μl of loading buffer (1 part 50 mM EDTA pH 8.0 in 50 mg/ml blue dextran solution to 5 parts denitized formamide).

**FISH detection and image analysis**

The regional assignment of the *Mgat3* allele was determined by fluorescence in situ hybridization (FISH) to normal mouse lymphocyte chromosomes counterstained with propidium iodide and 4',6-diamidino-2-phenylindol-dihydrochloride (DAPI) following published methods (Lichter et al., 1990; Boyle et al., 1992). Biotinylated probe (entire *NotI*-NotI mouse genomic *Mgat3* clone; see Figure 4) was prepared by nick translation and detected with antibody and avidin-FITC. Images of metaphase preparations were captured by a thermoelectrically cooled charge coupled camera (Photometrics, Tucson, AZ). Separate images of DAPI banded chromosomes (Heng and Tsui, 1993) and of FITC-targeted chromosomes were obtained and merged electronically using image analysis software (courtesy of Tim Rand and David Ward, Yale University) and pseudo-colored blue (DAPI) and yellow (FITC) as described previously (Boyle et al., 1992). The band assignment was determined by measuring the fractional chromosome length and by analyzing the banding pattern generated by the DAPI counterstained image (Francke, 1994; ISCN, 1978). The chromosomal localization was verified by double color FISH using a mouse extract is perhaps due to weak reactivity of E4-PHA with a nonbiotinized N-glycan-containing glycoprotein (Kobata and Yamahita, 1989, 1993) detected by L4-PHA.

**probe was labeled with digoxigenin (DIG) and detected with mouse anti-DIG antibody followed by DIG–anti-mouse antibody and rhodamine-anti-DIG. The chromosome 15 probe (med/Scn8a) was biotinylated and detected with FITC– avidin as described above.**

**RNA blot analysis**

RNA was prepared as previously described (Marth et al., 1985) and subjected to formaldehyde denaturing agarose gel electrophoresis. RNA was transferred onto nitrocellulose (Schleicher & Schuell) and hybridized to a random-primed radiolabeled DNA probe with Klenow. The *Mgat3* probe consisted of a 1.8 kilobase *BamHI* fragment which encompassed the entire GlcNAc-TUI protein-coding sequence. Following hybridization and washing conditions as above, the filter was exposed to Kodak XAR-5 film at ~80°C for 72 h. Methods were as described previously (Marth et al., 1985).

**Mgat3 gene targeting and mouse production**

From a phage 129/SvJ mouse genomic library, an *Mgat3* containing clone was used to generate a targeting vector as follows. A 1.8 kilobase *BamHI* fragment which contained the single *Mgat3* protein coding sequence exon was cloned into the *BamHI* site of the pBluescript vector. The flanking 2.0 kilobase *BamHI* fragment and an 8.5 kilobase *Smal* fragment were subsequently cloned into the Xhol and XhoI sites of pBluescript, respectively. Ten micrograms of the targeting DNA construct was linearized by NotI digestion, purified by agarose gel electrophoresis, and introduced into the RI ES cells via electroporation. ES cells were plated on gelatin-coated culture plates and selected for 10 days with medium containing 150 μg/ml of active G418 (Life Technologies, Grand Island, NY). Homologous recombinants were initially detected by polymers chain reaction (PCR) using a thymidine kinase promoter and *Mgat3* allele specific primers Tk303: 5' TGCAAACCACAAGTCGATCCG 3' and GnTII: 5' CTGCTATTTAGGGAGGAGGGGAAAATTATTGGC 3' respectively. Positive PCR positive clones were subjected to Southern blotting by methods described (Marth et al., 1985) to confirm homologous recombining.
nation had occurred. The genomic probe used was a 0.6 kb fragment which resides adjacent to the targeted Mga3 allele and was isolated from the phage Mga3 genomic clone by NolI (from the phage cloning site) and Kpnl digestion. In ES cell clone 1H8 bearing the targeted Mga3 allele, 10 μg of supercoiled Cre expression plasmid were transiently transfected by electroporation. Cells were plated at low dilution, and 4 days later the transfectants were selected for resistance to ganciclovir (2 × 10⁻⁴ M) for 5 days. Following selection, DNA from ganciclovir resistant clones was isolated and tested for recombination by Southern blot analyses. The 0.23 kb IzAP probe was isolated from pl1015-lox-I by NolI digestion and contains two loxP sites flanked by polynucleotide sequence, isolated previously from pllox as a 18 kb HindIII-EcoRI fragment (Orban et al., 1992) prior to blunt-end ligation into the BamHI site of pl1015-ΔspH 1.

Chimeric mice were generated by microinjection of 8–10 ES cells into 3.5 day C57BL/6 blastocyst-stage embryos and implanted into pseudopregnant outbred albino foster mother recipients as previously described (Mezler et al., 1994). The mutated Mga3 allele was crossed into the C57BL/6 background for the production of heterozygous Mga3 mutant mice.

DNA isolation

Cells and tissues were incubated for 5 h or overnight in 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% SDS; and protease K (100 μg/ml) (Boehringer Mannheim). DNA was purified by one phenol-chloroform extraction, one chloroform extraction and ethanol precipitation.

GlcNAc-TIII enzyme assay

Tissues were homogenized by 20 strokes using a Dounce homogenizer with 25 mM 2-(N-morpholine) ethanesulfonic acid (MES) buffer pH 6.5 containing 1% Triton X 100. GlcNAc-TIII was assayed using a synthetic acceptor substrate, GlcNAc[ß-1-3→4-Omethyl-Man] (alpha-1–6)-GlcNAc(ß-1-2)-4-Omethyl-Man (alpha-1–3) Manß-O(CH₂)₃COCH₃ (Khan et al., 1994). This substrate cannot be acted on by GlcNAc-TII, GlcNAc-TIV, nor GlcNAc-TV and is a highly specific substrate for GlcNAc-TIII. The assay was performed in a total volume of 20 μl containing 0.3 mM acceptor substrate, 62.5 mM GlcNaC (to inhibit N-acetylglucosaminidases), 3 mM AMP (to inhibit breakdown of UDP-[14C]GlcNAc by pyrophosphatase), 10 mM MnCl₂, 0.125% Triton X-100, 0.1 M MES pH 6.5, 1.25 mM UDP-[14C]GlcNaC (10,000 d.p.m./μmol) and 2.5 μl crude tissue homogenate. After incubating the tubes at 37°C for 2 h, the mixtures were diluted with 0.5 ml of water and loaded on to Sep-Pak C₁₈ cartridges. The cartridges were washed with water (40–60 ml) to remove unreacted radiolabeled donor and buffer components. The bound radiolabeled product was eluted with 3 ml of methanol and quantitated by liquid scintillation counting in LKB 1209 Rackbeta instruments after addition of 15 ml of scintillation fluid.

E₄-PHA binding assay

Tissues from wild-type (+/+), heterozygous (+/-), and homozygous-null (-/-) mice were homogenized as described above for the GlcNAc-TIII enzyme assay. The homogenates were made 0.1 N in HCl and heated at 80°C for 60 min. following by neutralization with dilute NaOH to remove terminal sialic acid residues on E₄-phytohemagglutinin-agarose column. In Fukuoka et al., 1992 prior to blunt-end ligation into the Cre-encoding plasmid, containing 3.5 kb of the 5' end of the Mga3 gene, including exons 1 and 2 and 2.5 kb of the 3' untranslated region.

Hematology and serum chemistry analyses

Mice were anesthetized with methoxyflurane and bled from the tail vein. Blood was collected in EDTA-coated polypropylene tubes (Becton Dickinson). Automated differentials and blood chemistry were determined by a CELL-DYN 3500 (UCSD Medical Center, Hillcrest). Mice were anesthetized with 0.3 ml of 2.5% Avertin and blood was drawn via a heart puncture and allowed to clot for 30 min at room temperature. Sample was subjected to centrifugation and supernatant was transferred to a new tube. Serum was examined by a Kodak Ektachem700 Analyzer (UCSD Medical Center, Hillcrest).

References


