A novel β(1,6)-*N*-acetylglucosaminyltransferase V (GnT-VB)[1]

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Received 30 September 2003; accepted 5 October 2003

First published online 30 October 2003

Edited by Judit Ovádi

Abstract UDP-*N*-acetylglucosamine:α(1,6)-D-mannoside β(1,6)-*N*- acetylglucosaminyltransferase (GnT-V, Mgat5) functions in the biosynthesis of *N*-linked glycans and is transcriptionally upregulated by oncogene signaling. We report here the cloning and characterization of a human cDNA encoding a distinct enzyme with related substrate specificity, termed GnT-VB, which is predicted to have 53% similarity to the original amino acid sequence of GnT-V(A). Transient expression of GnT-VB cDNA in COS7 cells yielded significant increases of activity toward GnT-VA acceptors, including synthetic saccharides and *N*-linked glycopeptides, with some differences in specificity. Unlike GnT-VA, GnT-VB required divalent cation for full activity. EST databases showed expression of a 6 bp (+) splice isoform of GnT-VB; when expressed, this enzyme showed significantly reduced activity. CHO Lec4 cells, which do not express GnT-VA or B activity, lack synthesis of the *N*-linked β(1,6) branch, and do not bind L-phytohemagglutinin (L-PHA), were transfected with GnT-VB or GnT-VA; both then bound significant amounts of L-PHA, demonstrating that both enzymes synthesized *N*-linked β(1,6) branched glycans in vivo. Real-time polymerase chain reaction results showed that GnT-VB mRNA was highly expressed in brain and testis, with lesser levels in other tissues, while human GnT-VA showed a more general expression, but with low levels in brain and no expression in skeletal muscle.

Key words: Glycosyltransferase; UDP-*N*-acetylglucosamine: α(1,6)-D-mannoside β(1,6)-*N*-acetylglucosaminyltransferase; L-Phytohemagglutinin; *N*-Linked β(1,6) branch

1. Introduction

UDP-*N*-acetylglucosamine:α-6-D-mannoside β-1,6-*N*-acetylglucosaminyltransferase V (GnT-V or Mgat5) transfers *N*-acetylglucosamine (GlcNAc) to the C-6 position of the β1,6-linked mannosyl residue in the trimannosyl core structure of complex-type *N*-glycans to generate GlcNAc(β1,6)[GlcNAcβ1,2] mannose (Man) (α1,6) [1]. A large amount of information is available on the expression of the glycans synthesized by GnT-V because the lectin L-phytohemagglutinin (L-PHA) binds to the galactosylated product of the GnT-V reaction, namely, galactose (Gal) β(1,4)GlcNAcβ(1,6)Manα(1,6) [1,2]. Consequently, the binding of this lectin to tissue sections, cells or blots has been used to visualize this product, even after additional sugars, such as sialic acid, are added. Many studies have demonstrated the association of increases in L-PHA binding and GnT-V activity with increases in cell invasiveness, and in some cases metastatic potential, and with decreased patient 5-year survival rates [3–7]. The increase in GnT-V activity and its cell surface products during oncogenesis results from increased transcription driven by activation of the *ras-ets* signaling pathway [8,9]. We report here the cloning and characterization of an additional member of the GnT-V family, termed GnT-VB, which shows 41% amino acid identity and 53% similarity to the original GnT-V sequence, which will be referred to as GnT-VA.

2. Materials and methods

2.1. Polymerase chain reaction (PCR) cloning of GnT-VB

A BLAST search [10] of the expressed sequence tag (EST) database identified a number of cDNAs that showed homology to the pre-existing human GlcNAcT-V. A full-length open reading frame (ORF) of an apparently novel GnT was identified in the Celera database. The cDNA encoding the full-length ORF was obtained by PCR using brain cDNA (Clontech) and cDNA derived from the SK-N-SH cell line (ATCC) as templates. The initial cDNA strand was synthesized by SuperScript III transcriptase (Invitrogen) via priming nine random oligomers and an oligo-dT primer. A 1.9 kb DNA fragment encoding the N-terminal GnT-VB (1–585) was amplified by PCR using primers EcoRI-hVB-F1: 5′-ctcgaattcACGATGGCCCTTCCTG-CCCTCCTG-3′ and hVB-R1986: 5′-CTCTGAGTTGTTGTAGTC-GACTGT-3′. A 1.3 kb DNA fragment encoding a C-terminal portion of GnT-VB (305–783) was amplified using primers hVB-F1262: 5′-ACGGAGGAGTCCGGGGACGTGTT-3′ and NotI-hVB-R2485: 5′-ctcgcggccgcTCACAGACAGCCCTGGCACAAG-3′. These fragments were subcloned into the p3T vector (MoBiTec). To yield a full-length cDNA, both plasmids were digested with *Eco*RI and *Pml*I. A 1.9 kb DNA fragment encoding an N-terminal portion of GnT-VB from p3T-VB-Nter was ligated into *Eco*RI-*Pml*I-digested

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[1] Nucleotide sequence data will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB114297.

Abbreviations: GnT-V, UDP-*N*-acetylglucosamine:α(1,6)-D-mannoside β(1,6)-*N*-acetylglucosaminyltransferase; L-PHA, L-phytohemagglutinin; GlcNAc, *N*-acetylglucosamine; Man, mannose; Gal, galactose; EST, expressed sequence tag; ORF, open reading frame; PCR, polymerase chain reaction; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01234-1

p3T-VB-Cter. The full-length cDNA was subcloned into pCDNA3.1 (Invitrogen) via *Eco*RI and *Not*I restriction sites, named pCD-VB. Similarly, the GnT-VB 6 bp insert variant was also subcloned into pCDNA3.1, named pCD-VB(+)6bp, as was the GnT-VA ORF (pCD-VA).

2.2. Sequence data analysis

An alignment of sequences was constructed by the CLUSTAL W program, ver. 1.60 [11], and illustrated with MacBoxshade, which was also used to calculate sequence identity and similarity. Based on the amino acid sequence alignment, nucleotide alignment was re-constructed by the AAtoNuc program, which was a gift from its developer, Mr. T. Moriya.

2.3. Real-time PCR analysis of GnT-VB transcripts

Marathon ready cDNAs of various human tissues were purchased from Clontech. Oligonucleotide primers for GnT-VA, GnT-VB, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were designed with Primer3 software. The primer set for GnT-VA: the forward primer was 5′-GAGCAGATCCTGGACCTCAG-3′, and the reverse primer was 5′-GCTGTCATGACTCCAGCGTA-3′. The primer set for GnT-VB was: 5′-TGTTCTCCCAGCATCCCTAC-3′ and 5′-TGGCTGCTTCAAACTCCTCT-3′. The primer set for G3PDH was: 5′-CAATGACCCCTTCATTGACC-3′ and 5′-GATCTCGCTCCTGGAAGATG-3′. Real-time PCR was performed using the iQTM SYBR Green Supermix (Bio-Rad). The PCR conditions were 95°C for 30 s 1 cycle, followed by 40 cycles of 95°C for 10 s, 65°C for 30 s; 95°C for 1 min, and 80 cycles of 55°C for 1 min for melting curve analysis. Standard curves for GnT-VA, GnT-VB, and G3PDH cDNAs were generated by serial dilution of a pCD-VA or pCD-VB and a p3T vector containing the hG3PDH cDNA.

2.4. PCR-restriction fragment length polymorphism (RFLP) for detection of 6 bp insert isoform of GnT-VB

To identify the relative abundance of the alternatively spliced isoforms of GnT-VB, we performed a PCR-RFLP experiment using brain and neuroblastoma cDNAs. A 448 bp (or 454 bp for the 6 bp insert isoform) DNA fragment of GnT-VB (371–517) was amplified by primers 5′-CGGCACATGGGACTCTCCTTCAAGAAG-3′ and 5′-CCTTGCGCAGCAGCTGCTGAAACTCAGGC-3′. PCR products were digested by *Bpm*I, and subjected to electrophoresis using a 3% agarose gel. The PCR product of the 6 bp insert isoform was separated into two fragments, 283 and 171 bp, by *Bpm*I digestion.

2.5. GnT-V enzymatic assays toward synthetic oligosaccharide and N-linked oligosaccharides

The GnT-V activity assays were performed as described [8]. COS7 cells were transiently transfected with pCD-VA, pCD-VB, pCD-VB(+)6bp or pCDNA3.1 alone by the lipofectamine method (Invitrogen). The synthetic tri- and disaccharide acceptors were: GlcNAcβ1-2Manα1-2Glc-O-octyl (Chemica Alta, Canada) and GlcNAcβ1-2Manα1-2-O-octyl (a gift from Dr. O. Hindsgaul), respectively, both acceptors were used at a concentration of 0.4 mM per reaction. Biantennary and triantennary aminopyridine-labeled (PA) oligosaccharide acceptors were obtained from glycopeptides digested by thermolysin (Calbiochem) from bovine fibrinogen (Sigma) and bovine fetuin (Sigma) respectively as described [12]. Oligosaccharides were released with 2 units of *N*-glycanase in 0.05 ml K_2HPO_4 pH 7.5 for 20 h at 37°C, and then separated from the peptides using a Sep-Pak C18 cartridge eluted with 0.1% trifluoroacetic acid. The glycans were then coupled to aminopyridine by reductive amination [13]. Acceptor PA-oligosaccharides were prepared by desialylation (2 N acetic acid, 100°C for 45 min) and then degalactosylated by 18 h incubation with 1.5 U of β-galactosidase from *Aspergillus oryzae* (Sigma), in 0.1 M acetic acid adjusted to pH 4.5 with triethylamine and analyzed by high performance liquid chromatography (HPLC). The structure of the PA-oligosaccharide acceptors was confirmed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry and by HPLC using a TSK amide column (TosoBiosciences) [14], comparing the retention times of their fluorescent peaks with those of authentic bi- and triantennary *N*-linked standards (V-Labs). For the glycosyltransferase assays, the PA-asialo-agalacto-biantennary or triantennary oligosaccharide acceptors were used at a concentration of 15 μM or 10 μM respectively and the reaction products were separated by HPLC as described above. Integrations of the fluorescent peaks separated on HPLC were used to calculate the ratios of product to unreacted acceptor in each assay. These ratios were then used to estimate the relative glycosyltransferase activity in each reaction, where the activity of the cells expressing GnT-VA for each acceptor was considered to be 100%.

2.6. Flow cytometry and oligosaccharide analysis of Lec4 transfectants

Lec4 cells were transfected with pCD-VB, pCD-VA, or pCDNA3.1, and cells were selected by 1 mg/ml G418 (Cellgro). After harvesting, cells were washed two times in phosphate-buffered saline (PBS), then blocked in PBA (2% bovine serum albumin/0.01% azide/PBS) for 10 min at 4°C. After blocking, cells were stained with 10 μg/ml of biotinylated L-PHA (Sigma) in PBA for 30 min at 4°C, followed by washing with PBA. Washed cells were stained with phycoerythrin-conjugated streptavidin (Sigma) for 30 min at 4°C. Following additional washing (three times) and fixation with 2% formaldehyde in PBS for 10 min at 4°C, cells were washed twice with PBA, and analyzed using a FACSCalibur (BD Biosciences) flow cytometer. Cell pellets were sonicated in PBS containing 0.1% Triton X-100 and then incubated overnight at 37°C with 1.5% (w/v) of TPCK-treated trypsin (Pierce). After incubation, the resulting peptide mixture was boiled for 10 min and dried. *N*-Linked glycans were then released by PNGase F, labeled with 2-aminopyridine and analyzed by HPLC as described above.

3. Results and discussion

We identified a novel candidate cDNA for human GnT-VB using a BLAST search of EST databases with the GnT-VA sequence as a query, which predicted a 2349 bp full-length ORF. The EST data indicated that the GnT-VB cDNA was expressed in brain, fetal brain, dorsal root ganglion, spleen, and tumor cells, including neuroblastoma, liver adenocarcinoma, lung large cell carcinoma, skin melanoma, and an epithelioid carcinoma cell line from pancreas. We performed PCR to isolate a full-length cDNA using brain and neuroblastoma cDNAs that encoded an ORF with 782 amino acids. The GnT-VB sequence does not contain a DXD/E motif usually considered as a potential sugar-nucleotide donor substrate binding site and/or divalent cation recognition site [15], similar to the lack of this motif in the GnT-VA sequence. The GnT-VB gene was found in the genome sequence (AC016168) that mapped to chromosome 17q25.3; by contrast, GnT-VA was located at chromosome 2q21.

Fig. 1 shows an amino acid sequence alignment of GnT-VB and GnT-VA. The nucleotide sequences showed 49% identity and 58% similarity, while the amino acid sequences were 41% identical and 53% similar. During cloning, a splice isoform of GnT-VB with a 6 bp insert between exon 10 and exon 11 (Fig. 1) was identified; both isoforms were represented in the EST database. To identify the relative abundance of the alternatively spliced isoforms of GnT-VB, we performed a PCR-RFLP experiment using brain and neuroblastoma cDNAs as templates. As shown in Fig. 2, neuroblastoma cDNA was cleaved into two fragments by *Bpm*I, which cleaved in the 6 bp insert sequence; however, brain cDNA was not cleaved. These data indicate, therefore, that brain cDNA contained the isoform of GnT-VB that lacked the 6 bp insert, by contrast to the neuroblastoma cells, which contained both isoforms.

The expression levels of GnT-VB and GnT-VA transcripts in human tissues were compared by real-time PCR analysis under conditions that did not distinguish between the splice isoforms of GnT-VB. Fig. 3 shows that the GnT-VB transcript was highly expressed in brain and testis. Spleen, thymus, and ovary expressed the GnT-VB transcripts at relatively lower amounts. Skeletal muscle showed low expression, but

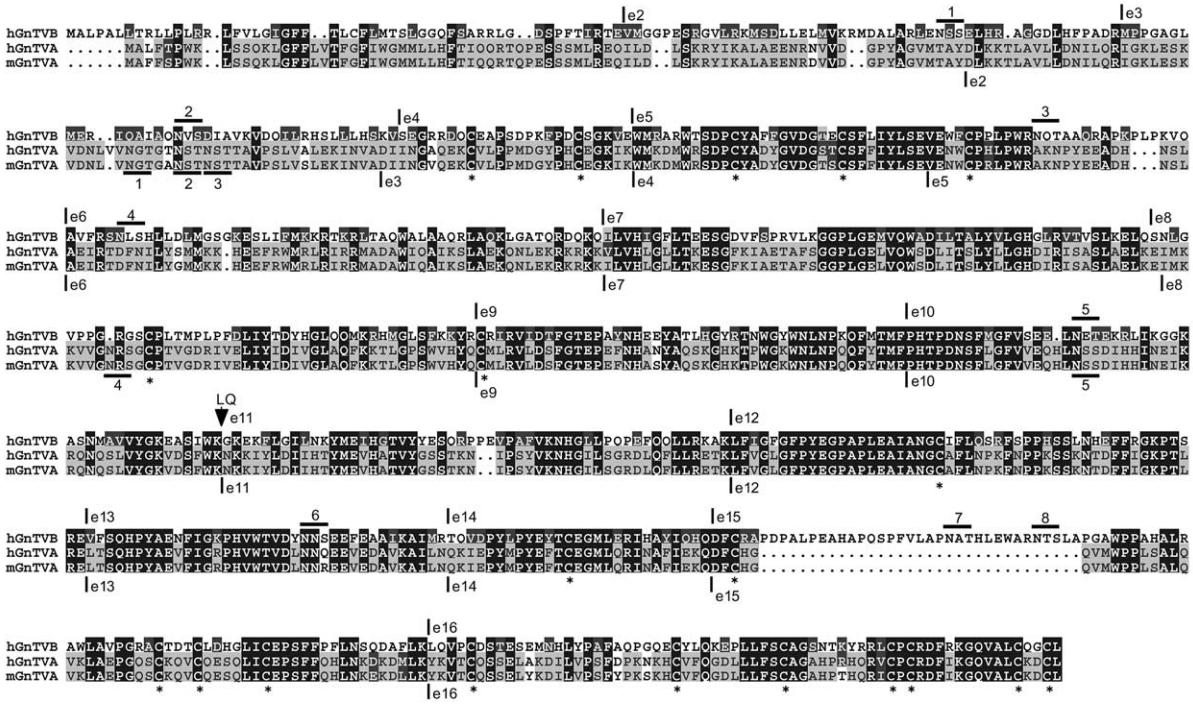

Fig. 1. Alignment of GnT-V amino acid sequences. Identical residues are boxed (black background with white character for all conserved amino acids, light gray background with black character for three conserved amino acid, and dark gray background with light gray character for similar amino acids). Putative *N*-glycosylation sites (GnT-VB) are indicated by a single line and number or a single line and number below the alignments (GnT-VA). Asterisks indicate the conserved cysteines. Predicted exon–intron junctions are indicated at the top for GnT-VB and at the bottom for GnT-VA. The 6 bp insert (2 amino acid insert, LQ) is indicated by the arrowhead.

these lower levels were accentuated due to high levels of G3PDH, which was used for normalization. By contrast, GnT-VA transcripts were expressed relatively ubiquitously, except for skeletal muscle, which was almost undetectable, while brain showed low expression levels.

To characterize enzymatic activity, the coding region for GnT-VB was transiently transfected into COS7 cells, as was a similar plasmid encoding GnT-VA, and an empty plasmid control, since COS7 cells express a low endogenous GnT-V activity. After 48 h, lysates of these transfectants were assayed for activity using a synthetic trisaccharide acceptor and conditions commonly used for assay of GnT-VA (Table 1). The activity of transiently transfected GnT-VA toward this acceptor is presented as 100%, and all other activities toward synthetic acceptors are given as relative values to this activity, since the assay conditions were originally optimized for GnT-VA. All activities were calculated by subtracting values obtained with mock-transfected cells, typically $<1\%$. GnT-VB showed little activity, but this activity was stimulated significantly in the presence of $MnCl_2$, by contrast to GnT-VA, which is active in EDTA and shows no stimulation by divalent cations. With $MnCl_2$, transiently expressed GnT-VB displayed about 33% of the GnT-VA activity toward the trisaccharide acceptor. GnT-VA preferred the trisaccharide acceptor over the disaccharide lacking the α(1,6)Man residue; however, GnT-VB displayed no difference in activity toward the two acceptors. Using PA-labeled asialo-agalacto-bi- and triantennary acceptors (lacking the β(1,6) branch), GnT-VB showed 40% and 33% activity, respectively (Table 1), compared to the activity of the GnT-VA enzyme toward each acceptor, which corresponds well with the relative values between GnT-VA and GnT-VB observed with the synthetic acceptors. The splice isoform GnT-VB(+)6bp showed about 14% of the activity of GnT-VA observed toward the trisaccharide acceptors in the presence of Mn^{2+}. GnT-VA showed 67% reduced activity toward the synthetic disaccharide,

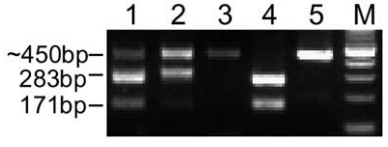

Fig. 2. PCR-RFLP for detection of 6 bp insert isoform. *Bpm*I restriction enzyme digestion of PCR fragments from brain and neuroblastoma cDNA. Lane 1, neuroblastoma IMR-32 cDNA; lane 2, SK-N-SH cDNA; lane 3, brain cDNA; lane 4, pCD-VB(+)6bp plasmid; lane 5, pCD-VB plasmid; and M, 100 bp marker.

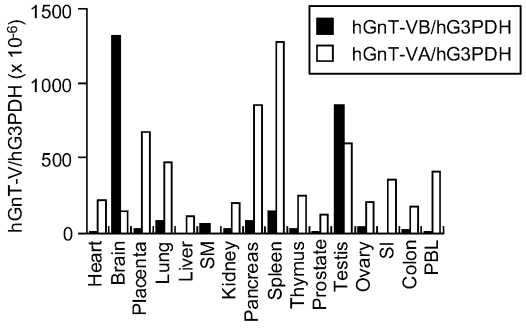

Fig. 3. Quantitative analysis of GnT-VA and transcripts in various human tissues by real-time PCR. The expression levels of GnT-VA and GnT-VB were each normalized to that of the G3PDH transcript. SM, skeletal muscle; SI, small intestine; PBL, peripheral blood leukocytes.

Table 1
Relative activity (%) of transiently transfected GnT-VA, GnT-VB, and the splice variant GnT-VB(+)6bp toward various acceptors

Substrate	Condition	GnT-VA	GnT-VB	GnT-VB(+)6bp
Trisaccharide	–	100[a]	15.3	9.1
	EDTA	104	9.2	8.6
	$MnCl_2$	80.8	33.0	13.7
Disaccharide	–	30.8	8.7	4.5
	EDTA	43.2	9.4	4.5
	$MnCl_2$	31.1	30.3	5.4
Asialo-agalacto triantennary	$MnCl_2$	100[b]	32.6	ND
Asialo-agalacto biantennary	$MnCl_2$	100[b]	39.9	ND

ND, not determined.
[a]The activity of GnT-VA without EDTA or $MnCl_2$ toward the trisaccharide acceptor was set to 100%, and values for all synthetic acceptors are relative to this value.
[b]The activity of GnT-VA toward each glycopeptide acceptor was set to 100%.

GlcNAcβ(1,2)Man-octyl compared to the trisaccharide; by contrast, GnT-VB transferred to the di- and trisaccharide substrates with equal activity, suggesting that GnT-VB may recognize a more minimal acceptor structure. Interestingly, the structure, GlcNAcβ(1,6)[GlcNAcβ(1,2)]Man-O-Ser/Thr, has been identified in rabbit brain, and its levels in this tissue are estimated to be about 15–20% of those of R-GalNAc-O-Ser/Thr [16]. Since GnT-VB shows high levels of expression in brain, it is intriguing to speculate that one of the native substrates for GnT-VB is the GlcNAcβ(1,2)Man-O-Ser/Thr structure, and that the product of this reaction may have a physiologically critical function in nerve and brain, as does the POMGnT1 product.

Lec4 cells lack GnT-V activity and cell surface *N*-linked GlcNAcβ(1,6) branches that are bound by L-PHA [17]. The pCD-VB plasmid and pCD-VA plasmid control were used to transfect Lec4 cells, followed by flow cytometry analysis using biotinylated L-PHA that was detected with fluorescently labeled streptavidin (Fig. 4). The results showed that both GnT-VB and GnT-VA expression resulted in similar levels of L-PHA binding and demonstrated that when expressed in vivo, GnT-VB is capable of synthesizing β(1,6) branched *N*-glycans recognized by L-PHA. The presence of tetraantennary *N*-linked structures in the transfected cells was confirmed by preparing glycopeptides from both transfectants, releasing the *N*-linked structures with *N*-glycanase, labeling them with 2-aminopyridine, and subjecting them to amino-based HPLC that separates bi-, tri-, and tetraantennary *N*-glycans whose structures were confirmed by MALDI-TOF mass spectrometry. A fluorescent glycan peak eluting identically to a tetraantennary structure was detected in GnT-VA and B transfectants, but not in mock-transfected cells, demonstrating that GnT-VB can synthesize in vivo a tetraantennary structure with a β(1,6) branched mannose (data not shown).

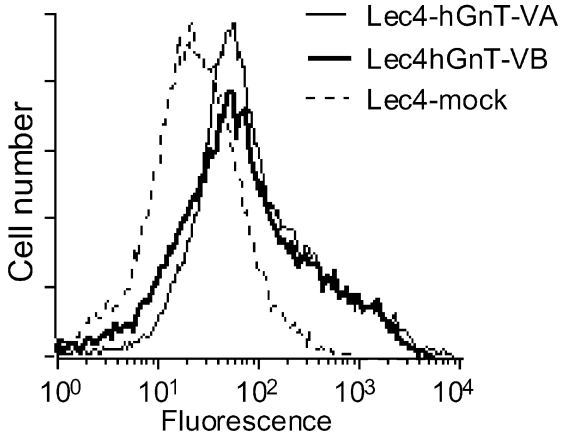

Fig. 4. Flow cytometry analysis of transfected Lec4 cells. Lec4 cells were transfected with the expression vector containing either: hGnT-VA (thin line), hGnT-VB (bold line), or with no insert (dotted line). Cells were incubated with biotinylated L-PHA, followed by fluorescently labeled streptavidin.

Our results demonstrate, however, that both in vivo and in vitro GnT-VB is clearly capable of synthesizing *N*-linked β(1,6) branched glycans. Because GnT-VB transcript expression levels are very high in brain relative to GnT-VA, these results suggest that some of the *N*-linked β(1,6) branched structures found in brain may be products of GnT-VB. In addition, two neuroblastoma cell lines showed high levels of the transcripts of both splice variants of GnT-VB, and EST databases revealed that several other tumor-derived cells showed their expression. Since GnT-VA is transcriptionally activated by oncogene signaling pathways, it is critical to determine if GnT-VB expression shows similar up-regulation during oncogenesis, particularly in tumors of neural origin.

4. Note added in proof

Inamori et al. (J. Biol. Chem., in press) have recently reported the sequence corresponding to the +6 bp splice isoform of GnT-VB, but termed the enzyme encoded by this sequence 'GnT-IX'. The lack of observable stimulation of enzyme activity by Mn^{2+} using a soluble form of the enzyme toward an asialo-agalacto biantennary acceptor is likely due to the inherently low activity of the +6 bp splice isoform compared to the −6 bp isoform (Table 1). Human brain appears to express only the −6 bp isoform (Fig. 2).

Acknowledgements: We are very grateful to Dr. Kelly Moremen for helpful discussions, to Dr. Ole Hindsgaul for providing synthetic substrates, and to Dr. Pamela Stanley for the Lec4 cells. This research was supported by NCI CA 064462 and NCRR P41RR018502.

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