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UDP-GlcNAc concentration is an important factor in the biosynthesis of β1,6-branched oligosaccharides: regulation based on the kinetic properties of *N*-acetylglucosaminyltransferase V

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Human β1,6-*N*-acetylglucosaminyltransferase V (GnT-V) was expressed by baculovirus-insect cell system, and the purified recombinant enzyme was kinetically characterized. The data obtained were used to establish the kinetic basis of the substrate specificity toward donor nucleotide sugars, and also revealed that K_m values for the donors are much higher compared to those of other GlcNAc transferases, the kinetic properties of which have been reported. Because this exceptionally higher K_m suggests that GnT-V is physiologically present at far from saturated conditions, it would appear that the production of β1,6-branched oligosaccharide, which is formed by GnT-V, could be regulated *in vivo* by the concentration of the donor, UDP-GlcNAc, as well as the expression levels of the enzyme. When B16 melanoma cells, which express high levels of GnT-V, were incubated with GlcNAc, the β1,6-branched oligosaccharide levels were increased, as judged by a lectin blot analysis, in conjunction with an increase in intracellular UDP-GlcNAc. These findings suggest that the level of UDP-GlcNAc can be a critical factor in the production of β1,6-branched oligosaccharides, for example, by tumor cells, which have been thought to be closely associated with tumor progression and metastasis.

Key words: donor specificity/kinetic analysis/ *N*-acetylglucosaminyltransferase V/recombinant enzyme/ UDP-GlcNAc

Introduction

It is a known fact that levels of the *N*-glycans with a core β1,6-branch are frequently increased in many malignant tumors, and it has been suggested that the increase is closely associated with tumor progression (Dennis and Laferte, 1989; Demetriou *et al.*, 1995, 2001; Granovsky *et al.*, 2000). This oligosaccharide structure is formed via the reaction by β1,6-*N*-acetylglucosaminyltransferase V (EC 2.4.1.155; GnT-V) (Brockhausen *et al.*, 1988; Cummings *et al.*, 1982) with UDP-GlcNAc as the glycosyl donor. Human GnT-V was purified from the conditioned medium of human lung carcinoma cell line, and its cDNA has then been cloned (Gu *et al.*, 1993; Saito *et al.*, 1994).

Some divalent metal-requiring GlcNAc transferases, such as GnTs-I (Nishikawa *et al.*, 1988), -III (Nishikawa *et al.*, 1992; Ihara *et al.*, 1993), -IV (Oguri *et al.*, 1997; Yoshida *et al.*, 1998), and -VI (Taguchi *et al.*, 2000; Sakamoto *et al.*, 2000), have the so-called DxD motif, which is thought to be involved in Mn^{2+} ion binding and catalysis (Busch *et al.*, 1998; Wiggins and Munro, 1998; Breton and Imberty, 1999). On the other hand, GnT-V does not appear to require divalent cation for its transfer reaction, as is also the case for other β1,6GlcNAc transferases. A common sequence motif involved in the catalytic mechanism has not been identified in this class of glycosyltransferase, because no significant sequence homology has not been found among the β1,6GlcNAc transferases. Therefore considerably less is known about the mechanism of GnT-V compared to other GlcNAc transferases in terms of chemical and structural bases, in spite of the clinical importance of GnT-V.

It is generally believed that GnT-V is involved in cancer metastasis, and, as a result, the development of potent and specific inhibitors has been attempted (Kanie *et al.*, 1993; Brockhausen *et al.*, 1995; Lu *et al.*, 1996). These inhibitors have been designed so as to mimic the acceptor oligosaccharide, probably because substrate specificity with respect to the acceptor has been intensively examined. However, the design of more potent inhibitors, such as transition analogs and slow-binding inhibitors, must await the elucidation of the catalytic mechanism. Because some major chemical reactions, specifically cleavage and reformation of bonds and inversion of configuration, during the transfer reaction occurs at a position C-1 of the transferred monosaccharide in the donor, it would be desirable to investigate the enzymatic properties of GnT-V in terms of its action with respect to the donor substrate, for example, structural requirements of the donor nucleotide-sugar for enzyme action, which would be expected to be involved in the catalytic mechanism.

In addition, though a detailed analysis of the properties of GnT-V would be useful for characterization, it may also provide valuable information as to whether GnT-V actually participates in the biosynthesis of the oligosaccharides in living cells. For example, it is possible that in some cells the production of β1,6-branched oligosaccharides might be unexpectedly low, even though the expression and activity of GnT-V are significantly high. A more detailed analysis could

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explain this possible discrepancy and would provide a more clear explanation of the relation between tumor progressions, the content of β1,6-branched oligosaccharides, and the expression or activity of the enzyme. Considering the importance of GnT-V and its product in tumor progression, such findings would be expected to be clinically useful.

In the present study, we report on the preparation of a poly-histidine-tagged soluble form of human GnT-V proteins using a baculovirus–insect cell expression system and kinetic analyses using various donor nucleotide-sugar derivatives to elucidate the structural requirements of the transferred monosaccharides. We show that not only UDP-GlcNAc but also UDP-Glc and TDP-Glc serve as donor substrates for GnT-V and that the specificity toward the nucleotide portion of the donor can be attributed to the catalytic step rather than the binding step. We also demonstrate that the *in vivo* level of UDP-GlcNAc is a critical factor in the production of β1,6-branched *N*-glycans.

Results

A baculovirus–insect cell system was used for the production of recombinant GnT-V. The enzyme was expressed in a fused form with a signal peptide derived from a baculoviral protein, gp67, to permit its efficient secretion into the medium, and was tagged with a His × 6 sequence at a C-terminus to allow purification using a metal-chelating Sepharose. In our previous study, we found that the cytoplasmic, transmembrane, and stem domains are not required for enzyme activity and that these regions could be replaced by other sequences (Sasai *et al.*, 2001). On the other hand, the deletion of the C-terminal 12 amino acid residues led to a complete loss of activity (data not shown), indicating that the C-terminal region is required for activity. Elimination of *N*-glycans by treatment with tunicamycine had no effect on activity (data not shown), suggesting that sugar chains are not required for activity. Therefore, it is more likely that differences in the sugar chain structures between host cells, mammalian and insect cells, have no effect on enzymatic properties. Two types of GnT-V proteins derived from baculovirus-insect cell system (BVV), BVVΔ73 and BVVΔ188, were prepared in this study, as shown in Figure 1A, and purified using a Ni^{2+}-immobilized Sepharose. For the respective proteins, 68 μg and 200 μg of the purified enzymes were obtained from 100 ml of medium. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis showed protein bands with M_r values of 85,000 and 70,000, respectively (Figure 1B, lanes 1 and 2), both of which were found to be His-tagged GnT-V proteins, as indicated by immunoblot analysis using an anti-GnT-V antibody and anti-polyhistidine antibody (Figure 1B, lanes 3–6). The specific activities of the purified BVVΔ73 and BVVΔ188 were determined to be 220 nmol/h/mg and 240 nmol/h/mg, respectively, using the standard assay.

The purified recombinant enzymes were subjected to kinetic analysis. When the activity was assayed for BVVΔ188 using various concentrations of 2-aminopyridine (PA)-labeled agalacto biantennary oligosaccharide (GnGn-bi-PA), in the presence of 40 mM UDP-GlcNAc, the enzyme reaction was not saturated even at concentrations as high as 400 μM (Figure 2). As indicated by a double reciprocal plot, it appears that the

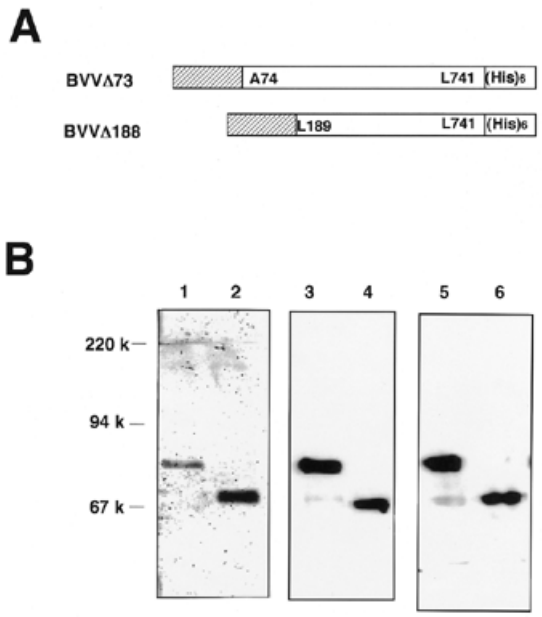

Fig. 1. Production of recombinant GnT-V proteins. (**A**) A schematic representations of constructs for two forms of human GnT-V are shown. The signal peptide derived from a baculoviral protein, gp67 (shaded boxes) were N-terminally fused to Ala-74 or Leu-189 of GnT-V. The enzymes were also tagged with a (His)6 sequence at the C-terminus. (**B**) BVVΔ73 (lanes 1, 3, and 5) and BVVΔ188 (lanes 2, 4, and 6) were analyzed by 8% SDS-gel. Protein bands were visualized by silver staining (lanes 1 and 2) and by immunoblotting using an anti-GnT-V antibody (lanes 3 and 4) and an anti-polyhistidine antibody (lanes 5 and 6).

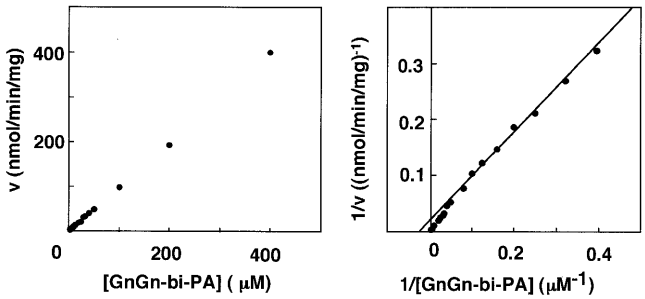

Fig. 2. Kinetic properties of GnT-V with respect to the acceptor PA-sugar chain. GnT-V activity was measured using the BVVΔ188 enzyme with various concentrations of GnGn-bi-PA in the presence of 40 mM UDP-GlcNAc. Left and right panels show [S]-v plot and 1/[S]-1/v plot, respectively.

enzyme is activated by the acceptor oligosaccharide in the range of high acceptor concentrations (40–400 μM), and thus the down curvature was found to be due to "substrate activation" kinetics. The K_m values for acceptors have been determined using a purified human enzyme (Gu *et al.*, 1993), partially purified rat enzyme (Korczak *et al.*, 2000), and a crude enzyme prepared from baby hamster kidney cells (Palcic *et al.*, 1990), and the values seemed to be relatively high. Substrate activation kinetics, such as the present study, have not been described in these earlier studies, and it is possible that the K_m values might

have been overestimated. With respect to the donor substrate, on the other hand, the reaction followed typical Michaelis-Menten-type kinetics. The concentrations of the acceptor in the linear portion of the double reciprocal plot were used to determine the kinetic parameters. As a result, the K_m values for the donor, UDP-GlcNAc, were 4.6 and 4.0 mM for BVVΔ73 and BVVΔ188, respectively, and the K_m for acceptors were 150 μM for both the recombinant enzymes. V_{max} values were 14 μmol/h/mg for BVVΔ73 and 13 μmol/h/mg for BVVΔ188. These results indicate that the kinetic properties of these enzymes are essentially the same and suggest that the protein region 74–188 is not involved in enzyme activity. When the apparent K_m values, which were obtained for either one of the donor and acceptor with the fixed concentration of the other substrate, were compared the recombinant enzyme produced by the insect cells is almost indistinguishable from the native rat kidney enzyme (Shoreibah *et al.*, 1992) and recombinant enzyme expressed in mammalian cells (Zhang *et al.*, 1997; Korczak *et al.*, 2000). Therefore, it seems unlikely that the use of baculovirus-insect cell system affects the kinetic properties of GnT-V.

To explore the substrate specificity toward donor nucleotide-sugars, enzyme activities were assessed using various UDP-sugars. The purified BVVΔ188 was incubated with 10 μM GnGn-bi-PA and 2 mM UDP-sugars for 10 h, and the products were analyzed by reversed-phased high-performance liquid chromatography (HPLC) (Figure 3). In the chromatographic separation, GnGn-bi-PA, used as the acceptor substrate, was eluted at 17 min, whereas the peak for the corresponding β1,6-branched agalactotriantennary sugar, to which the GlcNAc had been transferred, was found at 11 min. In the incubation with the natural donor, UDP-GlcNAc, no unreacted substrate remained due to the much faster reaction rate. When the enzyme was incubated with UDP-Glc, the reactions gave a product, which had retention time of 14 min. However, no product was found in the reaction with UDP-GalNAc and UDP-Gal, even if the reactions were conducted for a longer time, up to 160 h. This suggests that the configuration at the 4-hydroxyl group is of critical importance in the reaction of GnT-V. To confirm the transfers of Glc by GnT-V, the reaction product was collected and analyzed by electrospray ionization mass spectrometry (ESI-MS). As shown in Figure 4, MS analyses showed that the *m/z* values for $(M+H)^+$ were 1395.4 for the substrate agalacto biantennary (GnGn-bi-PA), 1598.4 for the product (which was produced by the reaction with the natural donor), and 1557.5 for the product from the reaction with UDP-Glc. This indicates that GnT-V is capable of catalyzing the transfer of Glc at a significant rate.

For an investigation of the involvement of the nucleotide portion of the donor in the reaction, we examined the issue of whether nucleotide-sugars with a nucleotide portion that differs from UDP could serve as a donor substrate for GnT-V. Because Glc can be transferred by the action of GnT-V, an investigation using a variety of nucleotide-Glc derivatives would allow the contribution of a nucleotide moiety to the transfer reaction by this enzyme to be evaluated. As shown in Figure 5, the activity assay indicated that the incubation of GnT-V with ADP-, CDP-, and GDP-Glc resulted in no product being formed, whereas TDP-Glc is active as the glycosyl donor as well as UDP-Glc. These results demonstrate that the recombinant GnT-V is capable of utilizing both UDP- and TDP-sugars, suggesting that structural features, which are common to uridine and thymidine but not to cytidine, play a role in the GnT-V-catalyzed glycosyl transfer.

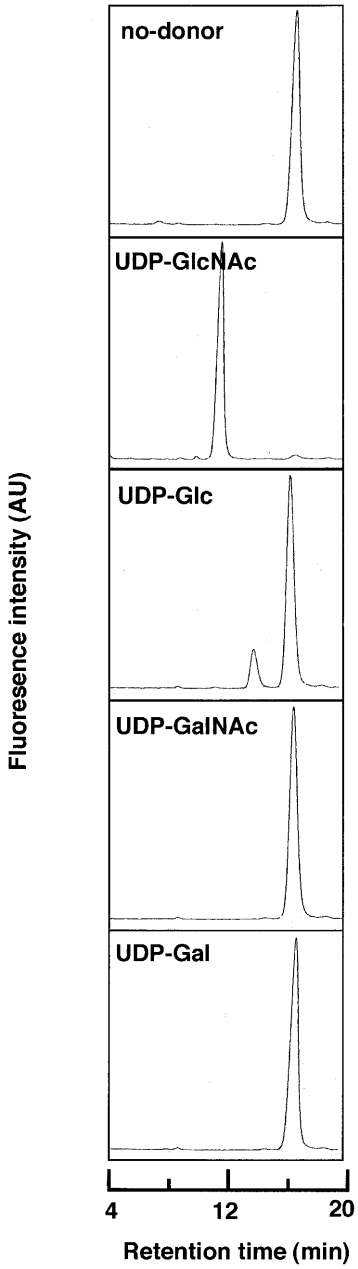

Fig. 3. Detection of GnT-V activities using various UDP-sugars. The purified GnT-V (BVVΔ188) was incubated with various UDP-sugars (2 mM) in the presence of 10 μM GnGn-bi-PA. The oligosaccharide peak with an elution time of 17 min represents unreacted substrate.

The elucidation of the kinetic factors associated with the distinct transfer rates on which the donor substrate specificity of GnT-V may be based would be important in understanding the mechanism underlying the specificity. To explore the kinetic basis for the donor substrate specificity of GnT-V, kinetic analyses of the reactions involving UDP-GlcNAc, UDP-Glc, and TDP-Glc were carried out. As shown in Figure 6, the reciprocal plots suggest that the reaction by GnT-V is

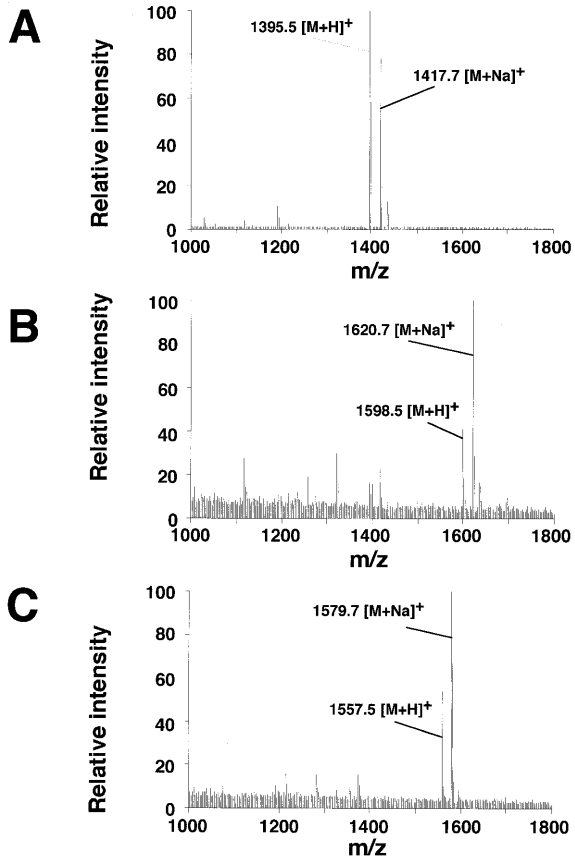

Fig. 4. ESI-MS analysis of the oligosaccharide products from the reactions of GnT-V. MS spectra of the products from the reactions with UDP-GlcNAc (**B**) and UDP-Glc (**C**) are shown with the spectrum of the substrate oligosaccharide (**A**). Detailed conditions for the analysis are described under *Materials and methods*.

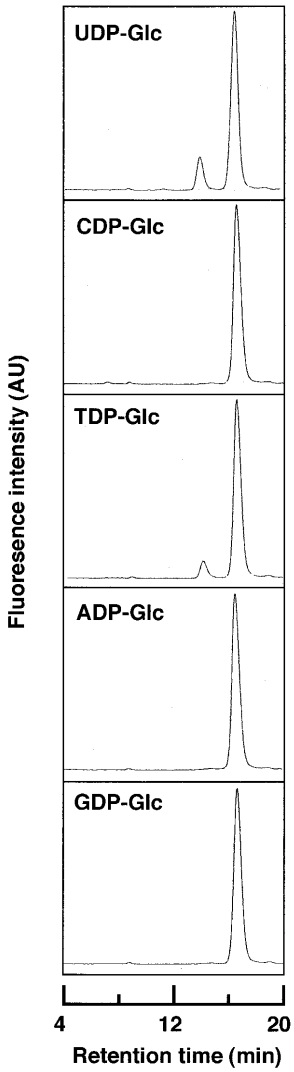

Fig. 5. Reactions with various nucleotide-Glc derivatives. The purified GnT-V was incubated with 2 mM various nucleotide-Glc derivatives as a donor in the presence of 10 μM of GnGn-bi-PA. The oligosaccharide peaks at 14 min and 17 min indicate Glc-transferred oligosaccharide and unreacted substrate, respectively.

consistent with a sequential mechanism in all these cases. From the secondary plots, the V_{max} values and the acceptor K_m values were obtained, and the relevant kinetic parameters are summarized in Table I. The K_m values for the donors and the acceptor were found to be essentially the same, and therefore it appears that the 2-*N*-acetyl group in monosaccharide portion and the 5-methyl group in the pyrimidine ring play no essential role in the binding of the donor. On the other hand, the V_{max} values in the reactions with UDP-Glc and TDP-Glc are 5% and 0.5%, respectively, compared to that for the natural donor, UDP-GlcNAc. These results suggest that the specificity toward the donor is largely dependent on the catalytic step rather than on the binding of the substrate.

On the basis of catalytic efficiency (k_{cat}/K_m for the donor), the specificity of GnT-V toward UDP-GlcNAc was estimated to be about 16 times higher than that of UDP-Glc, and this difference in the specificity can be attributed to the effect of the presence of the 2-*N*-acetyl group in the monosaccharide portion of the molecule. This property is much different from the case of GnT-III, whose k_{cat}/K_m value with respect to UDP-GlcNAc was more than 2000 times higher than that of UDP-Glc (Ikeda *et al.*, 2000). As indicated by the comparison of k_{cat}/K_m between UDP-Glc and TDP-Glc, the action of GnT-V is 30 times more specific for the UDP-sugar.

As shown in the kinetic analysis, the K_m value of GnT-V for the donor is 4.0 mM (Table I) and seems to be relatively larger, compared to other GlcNAc transferases (Szumilo *et al.*, 1987; Nishikawa *et al.*, 1988; Ikeda *et al.*, 2000). Furthermore, it would be more probable that this high K_m value is sufficiently higher than the intracellular or intra-Golgi concentration of UDP-GlcNAc (Murphy *et al.*, 1973; Waldman and Rudnick, 1990), therefore it would be expected that the enzyme responds to variations in the donor concentration in a linear manner. Under these conditions, the concentration is clearly a critical regulating factor in the biosynthesis of β1,6-branched oligosaccharides.

To investigate the effect of the UDP-GlcNAc concentration *in vivo* on the production of this type of sugar chain, GlcNAc

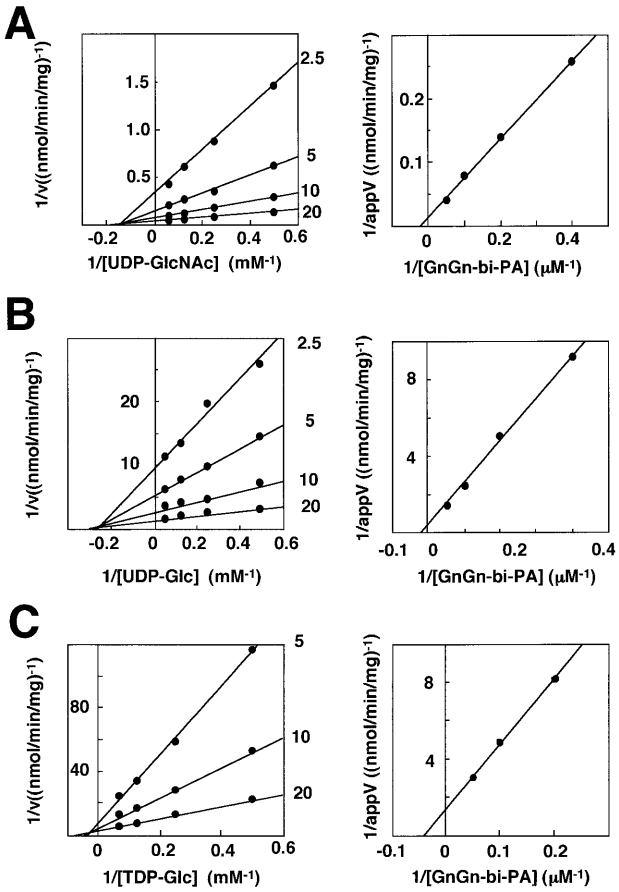

Fig. 6. Kinetic analysis of the GnT-V reactions involving UDP-GlcNAc, UDP-Glc, and TDP-Glc as the glycosyl donors. Left panels show the primary plots; acceptor concentrations for plot sets are expressed in μM and indicated on the left of the panels. Right panels show the secondary plots, in which reciprocal values of app V_{max} obtained from the primary plots are plotted as the function of 1/[acceptor].

was added to the culture of mouse B16 melanoma cells, which express a high level of GnT-V (Yoshimura *et al.*, 1995; Taniguchi *et al.*, 2000), to increase the intracellular UDP-GlcNAc level. Because nucleotide-sugar transporters are known to be a class of antiporter, transport of cytoplasmic UDP-GlcNAc into the lumen of the Golgi depends on the electrochemical potential differences of both UDP-GlcNAc and UMP across the membrane. Therefore, although the intra-Golgi level cannot be determined, it is conceivable that the intra-Golgi level of UDP-GlcNAc reflects the increase in the cytoplasmic level. The cells treated with sugars were analyzed by an UDP-GlcNAc determination and a lectin blot (Figure 7). As a result of the addition of 20 mM GlcNAc into the culture medium, the amount of UDP-GlcNAc was increased four times larger (Figure 7A). When the cells were analyzed by SDS–PAGE followed by Coomassie brilliant blue staining and carbohydrate staining, essentially no differences were found in the staining patterns (Figure 7C). In addition, the addition of GlcNAc also had no effect on the activity of GnT-V (Figure 7B). However, in the lectin blot analysis with leukoagglutinating phytohemagglutinin (L-PHA), which recognizes sugar chains with a core β1,6-branch, an enhancement of signals was observed (Figure 7C). It may be possible that the L-PHA staining is sometimes affected by the substitution and different linkages of β1,6-branch. However, because the addition of GlcNAc to the transfected GnT-V-overexpressing cells led to no alteration in the lectin binding, this treatment does not appear to cause such a structural alteration (data not shown). Therefore, it is suggested that the increase in the L-PHA binding results from the increased levels of β1,6-branch rather than other structural changes. On the other hand, the addition of 20 mM glucosamine did not affect the amount of UDP-GlcNAc and the reactivity toward L-PHA (Figure 7A, C). The same treatment of the GnT-III-transfected B16 cells did not increase the levels of bisected sugar chains, the products of GnT-III, as probed by E-PHA, a lectin that is specific for bisected oligosaccharides (data not shown). The treatment with 20 mM of the monosaccharides did not affect the viability of the cells. Thus these results suggest that the increase in intracellular UDP-GlcNAc level facilitates the *in vivo* production of β1,6-branched sugar chains without an increase in GnT-V activity.

Discussion

In this study, human GnT-V was expressed with a baculovirus–insect cell system, and the kinetic properties and the substrate specificity were analyzed using the purified recombinant enzymes. The kinetic analysis showed that the K_m for GnT-V with respect to the glycosyl donor, UDP-GlcNAc, is relatively high, compared to other GlcNAc transferases (Szumilo *et al.*, 1987; Nishikawa *et al.*, 1988; Ikeda *et al.*, 2000). The K_m value of GnT-V is also sufficiently higher than the intra-Golgi levels of UDP-GlcNAc, because the intracellular UDP-GlcNAc concentration appears to be as low as 100 μM (Murphy *et al.*,

Table I. Kinetic parameters for the transfer reactions by purified GnT-V

	Donor					
	V_{max}			k_{cat}/K_m		Acceptor[a]
Donor	(μmol/h/mg)	(%)	K_m(mM)	($M^{-1}s^{-1}$)	(%)	K_m(μM)
UDP-GlcNAc	13	(100)	4.0	45	(100)	150
UDP-Glc	0.59	(4.5)	2.9	2.8	(6.2)	230
TDP-Glc	0.058	(0.45)	9.3	0.083	(0.18)	130

[a]GnGn-bi-PA was used as an acceptor substrate.

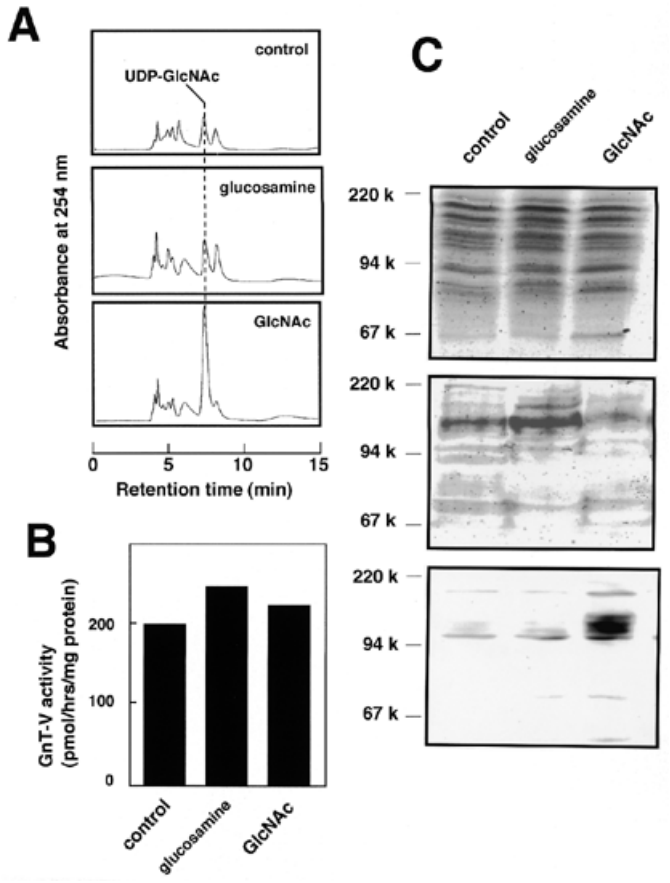

Fig. 7. Effects of addition of GlcNAc to B16-F10 cells. B16-F10 melanoma cells were cultured in the absence and presence of 20 mM monosaccharides for 72 h. (**A**) Determination of UDP-GlcNAc by anion exchange HPLC analysis. Elution was monitored by UV absorbance at 254 nm. Peaks of UDP-GlcNAc are indicated. (**B**) GnT-V activities in control and monosaccharide-treated cells. (**C**) Structural alteration in glycoprotein oligosaccharides. Cell homogenates were separated by SDS–PAGE and analyzed by Coomassie brilliant blue staining (top), oligosaccharide detection with DIG Glycan Detection kit (Boehringer) (middle), and lectin blot using L-PHA (bottom).

1973) and the intra-Golgi concentration was calculated, at least, to be lower than 1 mM (Waldman and Rudnick, 1990; Traynor *et al.*, 1996). It is possible that under these conditions, β1,6-branched oligosaccharides are produced in response to the changes in UDP-GlcNAc concentration in a linear manner. The donor K_m values for many GlcNAc transferases, which act on *N*-glycans, are probably comparable to the donor concentration or slightly higher; thus it is unlikely that the reaction rates of these GlcNAc transferases would be markedly increased in response to an increase in donor concentration. Therefore, even if UDP-GlcNAc levels are greatly increased in cells, it would not result in dramatic alterations in oligosaccharide structures on the cell surface. On the other hand, it would be expected that the core β1,6-branch structure is more formed as the UDP-GlcNAc level is increased, and, therefore, it is possible that an increase in β1,6-branched oligosaccharides could be caused without any enhancement in GnT-V expression. The present study suggests that the production of core β1,6-branched sugar chains by GnT-V is regulated not only by expression of the glycosyltransferase but also to a significant extent by the donor substrate level. This type of regulation may also contribute to variations and alterations of oligosaccharide structures.

It has been generally thought that the core β1,6-branched oligosaccharides are participating in cancer metastasis and malignancy, and, in this context, the potential importance of GnT-V has been emphasized (Dennis and Laferte, 1989; Demetriou *et al.*, 1995, 2001; Granovsky *et al.*, 2000). Hence the development of a potent and specific inhibitor of GnT-V has been attempted on the basis of specificity toward the acceptor substrates. This strategy was based on the definition of the minimal structural requirement for an oligosaccharide acceptor for GnT-V because the minimal requirements are different among the glycosyltransferases even though they share a common substrate. The results of investigation of donor substrate specificity suggest that the configuration at the C-4 position of the transferred monosaccharide is an essential factor in the action of GnT-V, as revealed by the inability of UDP-Gal and UDP-GalNAc to act as donor substrates. On the other hand, it was found that the catalytic efficiency toward UDP-Glc is about 6% of the values for UDP-GlcNAc, suggesting that the presence of a 2-*N*-acetyl group is not necessarily essential. In addition, it was shown that the thymidine nucleotide sugar also appears to be tolerated by GnT-V. These properties of GnT-V are different from those of another GlcNAc transferase, which is involved in core structure formation, such as GnT-III (Ikeda *et al.*, 2000), and the different characteristics indicate that the mechanism underlying the donor specificity of GnT-V is unique.

Because of the clinical importance of GnT-V and the β1,6-branched oligosaccharide, numerous efforts have been made to investigate correlations between GnT-V expression, to examine the content of the β1,6-branched sugar chain and malignant potential, and to develop specific inhibitors on the basis of acceptor specificity. However, as suggested by the present study involving kinetic analysis, the UDP-GlcNAc level also appears to be a particularly important factor in the regulation of β1,6-branched oligosaccharide formation, due to the unique kinetic properties of GnT-V. In some malignant cells in which UDP-GlcNAc levels are low, the levels of the β1,6-branched structure may be lower than expected, compared to GnT-V expression levels. Furthermore, a definition of the difference in the mechanism of the substrate specificity among glycosyltransferases with respect to donor as well as acceptor would be useful in designing more specific inhibitors.

Materials and methods

Materials

Restriction endonucleases and DNA-modifying enzymes were purchased from Takara, Toyobo, or New England Biolabs. Oligonucleotide primers were synthesized by Greiner Japan. GlcNAc and nucleotide-monosaccharaide derivatives were obtained from Sigma. Glucosamine was obtained from Nacalai Tesque. Antibodies were obtained from the following sources: anti-His monoclonal antibody (Qiagen); anti-GnT-V monoclonal antibody (a gift from Fujirebio); horseradish peroxidase–conjugated anti-mouse IgG antibody (Promega). Biotinated L-PHA

lectin was obtained from Seikagaku. Other common chemicals were obtained from Wako, Nacalai Tesque, and Sigma.

Site-directed mutagenesis

Site-directed mutagenesis was carried out according to the method of Kunkel (1985), for the creation of a poly-histidine tag (His × 6) at the C-terminus of the GnT-V. Briefly, the 3′ *Hin*d III–*Xba* I 0.7-kb fragment of human GnT-V cDNA was subcloned into a pBluescript SK+ (Stratagene). The uracil-substituted single-stranded template was prepared from *Escherichia coli* CJ236, which had been transformed by the plasmid. The uracil template was used with a synthetic oligonucleotide primer to replace the STOP codon by Gly and to extend the C-terminal sequence by Gly-Gly-His-His-His-His-His-His-(Stop). The oligonucleotide primer used in this study was 5′-AAA GAC TGC CTA GGG GGG GGA CAT CAT CAT CAC CAC CAC TAG CGG CCG GGG AAG ACA GTG-3′. The resulting mutation was verified by dideoxy sequencing using a DNA sequencer (DSQ-1000, Shimazu), as were the entire sequences that had been subjected to mutagenesis.

Transfer plasmids for baculovirus system

In this study, two forms of the recombinant proteins, namely, BVVΔ73 and BVVΔ188, were used. These represent C-terminus polyhistidine-tagged proteins that are fused to a cleavable signal peptide derived from baculoviral protein gp67. To introduce a polyhistidine tag at the C-terminus of the GnT-V, *Hind* III–*Xba* I fragments of expression plasmid for GnVd73 and GnV187, which are chimeric proteins with GnT-III (Sasai *et al.*, 2001), were replaced by the 3′ *Hin*d III–*Bam* HI fragment in which the tag sequence had been created as described (designed for GnVd73HIS and GnVd187HIS, respectively). For the preparation of BVVΔ73 protein, *Bsp* EI–*Eag* I fragment of GnVd73HIS was ligated to *Xma* I–*Eag* I site of a transfervector, pAcGP67-A vector (PharMingen). For the preparation of BVVΔ188 protein, expression plasmid for GnVd187HIS was digested by *Kpn* I and *Eag* I. The resulted fragment was treated with T4 polymerase and ligated in to *Sma* I site of a pAcGP67-A vector.

Expression of soluble recombinant hGnT-V in insect cells

Spodoptera frugiperda (Sf) 21 cells were maintained at 27°C in Grace's insect media (Gibco-BRL) supplemented with 10% fetal bovine serum, 3.33 g/L of yeastolate, 3.33 g/L of lactalbumin hydrolysate, and 100 mg/L of kanamycine. Recombinant viruses were manipulated as described previously (Murphy *et al.*, 1997). The purified transfer plasmid (1 μg) was cotransfected into 5×10^5 Sf21 cells with 10 ng of Baculo Gold DNA (PharMingen), which was used as *Autographa californica* nuclear polyhedrosis viral genome. The transfection experiments were carried out by the Lipofectin (Gibco-BRL) method (Felgner *et al.*, 1987), as described previously (Ikeda *et al.*, 2000). The recombinant virus, the titer of which was 5–10 multiples of infection, was infected to the cells (5×10^7, 80% confluent state) in a 175-cm^2 flask for 1 h. The medium was collected about 5 days postinfection for purification of the secreted GnT-V.

Purification of the recombinant enzyme

Cell debris in the culture medium was precipitated by saturated ammonium sulfate and pelleted by centrifugation. The pellet was dissolved in and dialyzed against 50 mM Tris–HCl (pH 8.0) and 200 mM NaCl. The dialyzed materials were applied to a Ni^{2+}-chelating Sepharose fast flow column (Amersham Pharmacia) equilibrated with 50 mM Tris–HCl (pH 8.0) containing 200 mM NaCl and 40 mM imidazole. The column was then washed thoroughly with 50 mM Tris–HCl (pH 8.0) containing 200 mM NaCl and 80 mM imidazole. The soluble GnT-V proteins were eluted from the column with 50 mM Tris–HCl (pH 8.0) containing 200 mM NaCl and 160 mM imidazole.

SDS–PAGE and immunoblotting

SDS–PAGE was carried out according to Laemmli (1970), and the protein bands were visualized using a silver-staining kit (Daiichi Pure Chemicals) or Coomassie brilliant blue. The separated proteins were electrophoretically transferred onto a PROTORAN (Schleicher and Schuell), followed by blocking with 5% skim milk. The resulting membrane was incubated with the first antibody. After washing, the membrane was then reacted with the anti-mouse antibody. The reactive protein bands were visualized by a chemiluminescence using an electrochemiluminescence system (Amersham-Pharmacia).

GnT-V activity assay and kinetic analysis

The GnT-V activity was assayed using a fluorescence-labeled oligosaccharide acceptor, as described previously (Taniguchi *et al.*, 1989; Sasai *et al.*, 2001). Cell homogenates (20 μg proteins) and purified enzyme (50 ng) were incubated at 37°C for 15 min with 10 μM GnGn-bi-PA as an acceptor and 2 mM UDP-GlcNAc as the donor in 125 mM MES-NaOH (pH 6.25) containing 200 mM GlcNAc, 0.5% Triton X-100 and 10 mM ethylenediamine tetra-acetic acid. The reaction was terminated by heating the mixture at 100°C for 2 min, and the sample was then centrifuged at 15,000 rpm for 5 min in a microcentrifuge. The resulting supernatant was analyzed by reversed-phase HPLC (Shimazu) using a TSKgel ODS-80TM (4.6 × 150, Tosoh). The solvent used was a 20 mM ammonium acetate buffer (pH 4.0), and the substrate and the product were isocratically separated. Fluorescence was detected with a fluorescence detector (RF-10AXL, Shimazu) at excitation and emission wavelengths of 320 nm and 400 nm, respectively. For kinetic analyses, the purified recombinant GnT-V proteins were incubated with various concentrations of GnGn-bi-PA and various nucleotide sugar derivatives.

ESI-MS

ESI-MS was carried out as described previously (Ikeda *et al.*, 2000) using an LCQ (Finnigan) quadrupole mass spectrometer. The PA-labeled oligosaccharide was dissolved in a 50% aqueous methanol and introduced into the ion source by direct infusion at a flow rate of 3 μl/min using a syringe pump integrated into system. ESI-MS spectra were obtained using the positive ion mode. The ion spray voltage and capillary voltage were 4.5 kV and 10 V, respectively, and capillary temperature was 200°C. Full scan spectra were obtained in the range of 1000–1800.

Cell culture

B16-F10 melanoma cells were routinely maintained at 37°C in Dulbecco's modified Eagle medium (Nikken) supplemented with 10% fetal calf serum (Gibco-BRL), 50 U/ml penicillin G,

and 50 μg/ml streptomycin under a humidified atmosphere of 95% air and 5% CO_2.

Monosaccharide treatment

When B16-F10 cells reached at approximately 50% confluence, monosaccharides, GlcNAc, or glucosamine were supplemented with normal culture media at the concentration of 20 mM. After 72 h, the extracted cellular proteins were subjected to SDS–PAGE, and the separated proteins were electrophoretically transferred onto a PROTORAN. The transferred membranes were blocked with 2% bovine serum albumin and incubated with biotin-labeled lectin. After washing, the membrane was reacted with an avidin-biotin complex with the peroxidase. The reactive glycoprotein bands were visualized by chemiluminescence using an ECL system (Amersham-Pharmacia). For the detection of sugars in glycoconjugates, the transferred membrane was treated with a DIG Glycan Detection Kit (Boehringer Mannheim) according to the manufacture's protocol.

Determination of the amount of cellular UDP-GlcNAc

B16-F10 cells were scraped and homogenized in 0.7 M perchloric acid, followed by microcentrifugation at 15,000 rpm. The resulting supernatants were neutralized with 5 M potassium carbonate and microcentrifuged at 15,000 rpm. The resulting supernatants, containing the extracted nucleotide-sugars, were separated on a Partisil SAX anion exchange HPLC column (4.6 × 250 mm) and quantified by UV absorption at 254 nm as described previously (Robinson *et al.*, 1995).

Protein determination

Protein concentrations were determined using the method described by Bradford (1976) using bovine serum albumin as a standard.

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Abbreviations

BVV, GnT-V proteins derived from baculovirus-insect cell system; ESI-MS, electrospray ionization mass spectrometry; GnGn-bi-PA, 2-aminopyridine-labeled agalacto biantennary oligosaccharide; GnT-V, *N*-acetylglucosaminyltransferase V; HPLC, high-performance liquid chromatography; PA, 2-aminopyridine; L-PHA, leukoagglutinating phytohemagglutinin; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Sf, *Spodoptera frugiperda*.

References

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

Breton, C. and Imberty, A. (1999) Structure/function studies of glycosyltransferases. *Curr. Opin. Struct. Biol.*, **9**, 563–571.

Brockhausen, I., Reck, F., Kuhns, W., Khan, S., Matta, K.L., Meinjohanns, E., Paulsen, H., Shah, R.N., Baker, M.A., and Schachter, H. (1995) Substrate specificity and inhibition of UDP-GlcNAc:GlcNAc β 1-2Man alpha 1-6R β 1, 6-*N*-acetylglucosaminyltransferase V using synthetic substrate analogues. *Glycoconj. J.*, **12**, 371–379.

Brockhausen, I., Carver, J.P., and Schachter, H. (1988) Control of glycoprotein synthesis. The use of oligosaccharide substrates and HPLC to study the sequential pathway for *N*-acetylglucosaminyltransferases I, II, III, IV, V, and VI in the biosynthesis of highly branched N-glycans by hen oviduct membranes. *Biochem. Cell Biol.*, **66**, 1134–1151.

Busch, C., Hofmann, F., Selzer, J., Munro, S., Jeckel, D., and Aktories, K. (1998) A common motif of eukaryotic glycosyltransferases is essential for the enzyme activity of large clostridial cytotoxins. *J. Biol. Chem.*, **273**, 19566–19572.

Cummings, R.D., Trowbridge, I.S., and Kornfeld, S. (1982) A mouse lymphoma cell line resistant to the leukoagglutinating lectin from Phaselusvulgaris is deficient in UDP-GlcNAc: α-D-mannoside β 1, 6 *N*-acetylglucosaminyltransferase. *J. Biol. Chem.*, **257**, 13421–13427.

Demetriou, M., Granovsky, M., Quaggin, S., and Dennis, J.W. (2001) Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature*, **409**, 733–739.

Demetriou, M., Nabi, I.R., Coppolino, M., Dedhar, S., and Dennis, J.W. (1995) Reduced contact-inhibition and substratum adhesion in epithelial cells expressing GlcNAc-transferase V. *J. Cell Biol.*, **130**, 383–392.

Dennis, J.W. and Laferte, S. (1989) Oncodevelopmental expression of GlcNAc β 1-6Man α 1-6Man β 1–branched asparagine-linked oligosaccharides in murine tissues and human breast carcinomas. *Cancer Res.*, **49**, 945–950.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., and Danielsen, M. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl Acad. Sci. USA*, **84**, 7413–7417.

Granovsky, M., Fata, J., Pawling, J., Muller, W.J., Khokha, R., and Dennis, J.W. (2000) Suppression of tumor growth and metastasis in Mgat5-deficient mice. *Nat. Med.*, **6**, 306–312.

Gu, J., Nishikawa, A., Tsuruoka, N., Ohno, M., Yamaguchi, N., Kangawa, K., and Taniguchi, N. (1993) Purification and characterization of UDP-*N*-acetylglucosamine: α-6-D-mannoside β 1-6*N*-acetylglucosaminyltransferase (*N*-acetylglucosaminyltransferase V) from a human lung cancer cell line. *J. Biochem. (Tokyo)*, **113**, 614–619.

Ihara, Y., Nishikawa, A., Tohma, T., Soejima, H., Niikawa, N., and Taniguchi, N. (1993) cDNA cloning, expression, and chromosomal localization of human *N*-acetylglucosaminyltransferase III (GnT-III). *J. Biochem. (Tokyo)*, **113**, 692–698.

Ikeda, Y., Koyota, S., Ihara, H., Yamaguchi, Y., Korekane, H., Tsuda, T., Sasai, K., and Taniguchi, N. (2000) Kinetic basis for the donor nucleotide-sugar specificity of β1, 4-*N*-Acetylglucosaminyltransferase III. *J. Biochem. (Tokyo)*, **128**, 609–619.

Kanie, O., Crawley, S.C., Palcic, M.M., and Hindsgaul, O. (1993) Acceptor-substrate recognition by *N*-acetylglucosaminyltransferase-V: critical role of the 4-hydroxyl group in β-D-GlcpNAc-(1-2)-α-D-Manp(1-6)-β-D-Glcp-OR. *Carbohydr. Res.*, **243**, 139–164.

Korczak, B., Le, T., Elowe, S., Datti, A., and Dennis, J.W. (2000) Minimal catalytic domain of *N*-acetylglucosaminyltransferase V. *Glycobiology*, **10**, 595–599.

Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. USA*, **82**, 488–492.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Lu, P.P., Hindsgaul, O., Compston, C.A., and Palcic, M.M. (1996) New synthetic trisaccharide inhibitors for *N*-acetylglucosaminyltransferase-V. *Bioorg. Med. Chem.*, **4**, 2011–2022.

Murphy, C.I., Piwnica-Worm, H., Grunwald, S., and Romanow, W.G. (1997) Maintenance of insect cell culture and generation of recombinant baculoviruses. In Ausubel, F.M., Brent, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.) *Current Protocol in Molecular Biology*. John Wiley and Sons, Chichester, UK, 16.10.1–16.10.17.

Murphy, G., Ariyanayagam, A.D., and Kuhn, N.J. (1973) Progesterone and the metabolic control of the lactose biosynthetic pathway during lactogenesis in the rat. *Biochem. J.*, **136**, 1105–1116.

Nishikawa, A., Ihara, Y., Hatakeyama, M., Kangawa, K., and Taniguchi, N. (1992) Purification, cDNA cloning, and expression of UDP-*N*-acetylglucosamine: β-D-mannoside β-1, 4*N*-acetylglucosaminyltransferase III from rat kidney. *J. Biol. Chem.*, **267**, 18199–18204.

Nishikawa, Y., Pegg, W., Paulsen, H., and Schachter, H. (1988) Control of glycoprotein synthesis: Purification and characterization of rabbit liver

UDP-*N*-acetylglucosamine:α-3-D-mannoside β-1, 2-*N*-acetylglucosaminyltransferase I. *J. Biol. Chem.*, **263**, 8270–8281.

Oguri, S., Minowa, M.T., Ihara, Y., Taniguchi, N., Ikenaga, H., Takeuchi, M. (1997) Purification and characterization of UDP-*N*-acetylglucosamine: α1, 3-D-mannoside β1, 4-*N*-acetylglucosaminyltransferase (*N*-acetylglucosaminyltransferase-IV) from bovine small intestine. *J. Biol. Chem.*, **272**, 22721–22727.

Palcic, M.M., Ripka, J., Kaur, K.J., Shoreibah, M., Hindsgaul, O., and Pierce, M. (1990) Regulation of *N*-acetylglucosaminyltransferase V activity: kinetic comparisons of parental, Rous sarcoma virus transferomed BHK, and L-phytohemagglutinin-resistant BHK cells using synthetic substrates and an inhibitory substrate analog. *J. Biol. Chem.*, **265**, 6759–6769.

Robinson, K.A., Weinstein, M.L., Lindenmayer, G.E., and Buse, M.G. (1995) Effects of diabetes and hyperglycemia on the hexosamine synthesis pathway in rat muscle and liver. *Diabetes*, **44**, 1438–1446.

Saito, H., Nishikawa, A., Gu, J., Ihara, Y., Soejima, H., Wada, Y., Sekiya, C., Niikawa, N., and Taniguchi, N. (1994) cDNA cloning and chromosomal mapping of human *N*-acetylglucosaminyltransferase V+. *Biochem. Biophys. Res. Commun.*, **198**, 318–327.

Sakamoto, Y., Taguchi, T., Honke, K., Korekane, H., Watanabe, H., Tano, Y., Dohmae, N., Takio, K., Horii, A., and Taniguchi, N. (2000) Molecular cloning and expression of cDNA encoding chicken UDP-*N*-acetyl-D-glucosamine (GlcNAc): GlcNAc β1-6(GlcNAc β1-2)-man α 1-R[GlcNAc to man] β1, 4 *N*-acetylglucosaminyltransferase-VI. *J. Biol. Chem.*, **275**, 36029–36034.

Sasai, K., Ikeda, Y., Tsuda, T., Ihara, H., Korekane, H., Shiota, K., and Taniguchi, N. (2001) The critical role of the stem region as a functional domain responsible for the oligomerization and Golgi localization of *N*-Acetylglucosaminyltransferase V: the involvement of domain homophilic interaction. *J. Biol. Chem.*, **276**, 759–765.

Shoreibah, M.G., Hindsgaul, O., and Pierce, M. (1992) Purification and characterization of rat kidney UDP-*N*-acetylglucosamine:α-6-mannoside β-1, 6-*N*-acetylglucosaminyltransferase. *J. Biol. Chem.*, **267**, 2920–2927.

Szumilo, T., Kaushal, G.P., and Elbein, A.D. (1987) Purification and properties of the glycoprotein processing *N*-Acetylglucosaminyltransferase II from plants. *Biochemistry*, **26**, 5498–5505.

Taguchi, T., Ogawa, T., Inoue, S., Inoue, Y., Sakamoto, Y., Korekane, H., and Taniguchi, N. (2000) Purification and characterization of UDP-GlcNAc: GlcNAcβ 1-6(GlcNAcβ 1-2)Manα 1-R [GlcNAc to Man]-β 1, 4-*N*-acetylglucosaminyltransferase VI from hen oviduct. *J. Biol. Chem.*, **275**, 32598–32602.

Taniguchi, N., Jain, S.K., Takahashi, M., Ko, J.H., Sasai, K., Miyoshi, E., and Ikeda, Y. (2000) Glycosyltransferses: cell surface remodeling and regulation of receptor tyrosine kinases-induced signaling. *Pure Appl. Chem.*, **71**, 719–728.

Taniguchi, N., Nishikawa, A., Fujii, S., and Gu, J.G. (1989) Glycosyltransferase assays using pyridylaminated acceptors: *N*-acetylglucosaminyltransferase III, IV, and V. *Meth. Enzymol.*, **179**, 397–408.

Traynor, A.J., Hall, E.T., Walker, G., Miller, W.H., Melancon, P., and Kuchta, R.D. (1996) Inhibition of UDP-*N*-Acetylglucosamine import into Golgi membranes by nucleotide monophosphates. *J. Med. Chem.*, **39**, 2894–2899.

Waldman B.C. and Rudnick, G. (1990) UDP-GlcNAc transport across the Golgi membrane: electroneutral exchange for dianionic UMP. *Biochemistry*, **29**, 44–52.

Wiggins, C.A. and Munro, S. (1998) Activity of the yeast MNN1 α-1, 3-mannosyltransferase requires a motif conserved in many other families of glycosyltransferases. *Proc. Natl Acad. Sci. USA*, **95**, 7945–7950.

Yoshida, A., Minowa, M.T., Takamatsu, S., Hara, T., Ikenaga, H., and Takeuchi, M. (1998) A novel second isoenzyme of the human UDP-*N*-acetylglucosamine:α1, 3-D-mannoside β1, 4-*N*-acetylglucosaminyltransferase family: cDNA cloning, expression, and chromosomal assignment. *Glycoconj. J.*, **15**, 1115–1123.

Yoshimura, M., Nishikawa, A., Ihara, Y., Taniguchi, S., and Taniguchi, N. (1995) Suppression of lung metastasis of B16 mouse melanoma by *N*-acetylglucosaminyltransferase III gene transfection. *Proc. Natl Acad. Sci. USA*, **92**, 8754–8758.

Zhang, N., Peng, K.C., Chen, L., Puett, D., and Pierce, M. (1997) Circular dichroic spectroscopy of *N*-acetylglucosaminyltransferase V and its substrate interactions. *J. Biol. Chem.*, **272**, 4225–4229.