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Kinetic Basis for the Donor Nucleotide-Sugar Specificity of β1,4-*N*-Acetylglucosaminyltransferase III[1]

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The kinetic basis of the donor substrate specificity of β1,4-*N*-acetylglucosaminyltransferase III (GnT-III) was investigated using a purified recombinant enzyme. The enzyme also transfers GalNAc and Glc moieties from their respective UDP-sugars to an acceptor at rates of 0.1–0.2% of that for GlcNAc, but Gal is not transferred at a detectable rate. Kinetic analyses revealed that these inefficient transfers, which are associated with the specificity of the enzyme, are due to the much lower V_{max} values, whereas the K_m values for UDP-GalNAc and UDP-Glc differ only slightly from that for UDP-GlcNAc. It was also found that various other nucleotide-Glc derivatives bind to the enzyme with comparable affinities to those of UDP-GlcNAc and UDP-Glc, although the derivatives do not serve as glycosyl donors. Thus, GnT-III does not appear to distinguish UDP-GlcNAc from other structurally similar nucleotide-sugars by specific binding in the ground state. These findings suggest that the specificity of GnT-III toward the nucleotide-sugar is determined during the catalytic process. This type of specificity may be efficient in preventing a possible mistransfer when other nucleotide-sugars are present in excess over the true donor.

Key words: *N*-acetylglucosaminyltransferase, bisecting GlcNAc, GnT-III, nucleotide-sugar, substrate specificity.

N-Acetylglucosaminyltransferase III (GnT-III) catalyzes the transfer of an *N*-acetylglucosamine (GlcNAc) moiety from a donor substrate, UDP-GlcNAc, to a core β-mannosyl residue of an *N*-linked oligosaccharide *via* a β1,4 linkage, resulting in the formation of a unique structure, a bisecting GlcNAc (*1*). The resulting sugar chain, which is referred to as a bisected oligosaccharide, does not serve as a substrate for α-mannosidase II and GnTs-II, -IV, and -V, and, thus, the formation of additional branches is inhibited (*1–6*). Thus, it is generally thought that GnT-III and its product play a regulatory role in the biosynthesis of complex and hybrid types of *N*-linked oligosaccharides. Since the addition of the bisecting GlcNAc also tends to prevent the elongation of branches in the oligosaccharides, the size of *N*-linked sugar chains is likely to be reduced in the case of a cell which expresses GnT-III (*7*). In addition to its importance in the biosynthesis of sugar chains, the significance of the bisecting GlcNAc has been implicated in a variety of other biological events (*8–16*).

It is known that the activity and gene expression of GnT-III are increased during hepatocarcinogenesis (*17–22*), and it has been suggested that this enhanced expression of GnT-III leads to the elevation of levels of the bisecting GlcNAc content in oligosaccharide moieties of glycoproteins which are produced in the liver, such as transferrin (*23*, *24*). The increase in the levels of the bisected sugar chain is often considered to be one of the "cancer-associated alterations" of sugar chains. GnT-III has been purified from rat kidney (*25*), and the cDNAs for the rat and human enzymes have been cloned, in an attempt to investigate the molecular basis of this structural alteration and the biological significance of the bisecting GlcNAc (*25*, *26*). The primary structure, which has been deduced from the cloned cDNA, reveals that GnT-III is a typical type II membrane protein, consisting of a short N-terminal cytoplasmic tail, a transmembrane domain, a stem region and a large catalytic domain. These structural features are quite similar to those of a large number of glycosyltransferases (*27*). However, no significant sequence homology has been found between GnT-III and other known glycosyltransferases, even though GnT-III shares either UDP-GlcNAc or acceptor oligosaccharides in common with many other GlcNAc transferases.

Substrate specificity with respect to the acceptor has been intensively examined in attempts to characterize the enzymatic properties of GnT-III. These studies have shown that: (a) high mannose type oligosaccharides are not active as substrates, and β1,2GlcNAc linked to α1,3Man, which is a product of GnT-I, is required for the action of GnT-III; (b) any agalacto form of the bi-, tri-, and tetra-antennary sugar chains is capable of serving as the acceptor substrate for GnT-III; (c) β1,4galactosylation of GlcNAcβ1-2Manα1-3

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Abbreviations: ESI, electrospray ionization; GnT, *N*-acetylglucosaminyltransferase; MS, mass spectrometry; PA, 2-aminopyridine.

branch inhibits the action of GnT-III; (d) an α1,6fucosyl residue at the innermost GlcNAc has no effect on the enzyme reaction (*2*). These characterizations of GnT-III in terms of the acceptor substrate specificity have permitted the structural requirements for the acceptor to be defined. Studies on the availability of the bisected sugar chains as the substrate toward α-mannosidase-II and other glycosyltransferases such as GnT-IV, GnT-V, and β1,4galactosyltransferase have established the enzymatic basis for the regulatory roles of the bisecting GlcNAc in the biosynthesis of *N*-glycans (*2–6*). It seems that the specificity toward acceptors is broader than that toward the donor, because only the products to which GlcNAc has been transferred have been identified in naturally occurring oligosaccharides.

In contrast to the detailed investigations of acceptor specificity, the structural requirements of the donor nucleotide-sugar in the reaction catalyzed by GnT-III are not known, since the substrate specificity of GnT-III toward the donor has not been explored to any extent. Therefore, the underlying mechanism for the highly specific enzyme action, with respect to GlcNAc still remains unclear. It is generally known that the specific binding that leads to the formation of the enzyme-substrate complex is one of the most critical steps in distinguishing a substrate from other compounds in the course of the enzyme reaction, and thus plays a critical role in substrate specificity. Although various nucleotide-sugars serve as glycosyl donors in the biosynthesis of oligosaccharides, many glycosyltransferases are capable of exclusively utilizing their own unique donor nucleotide-sugars, in spite of only subtle structural differences in the monosaccharide portions. Glycosyltransferases appear to strictly distinguish subtle structural differences, for example, a different configuration at the C-4 position, *i.e.*, UDP-GlcNAc *vis-a-vis* UDP-GalNAc. An investigation into the mechanism by which a particular nucleotide-sugar is discriminated from others and utilized as a donor for the transfer reaction represents one of the most important issues in the elucidation of the nature of substrate specificity, not only for GnT-III but for other glycosyltransferases as well. Specificity toward both donor and acceptor substrates represents a key factor in the strictly regulated construction of oligosaccharides, because the ordered actions of the glycosyltransferases, which collectively constitute the biosynthetic pathway, are at least in part dependent on their substrate specificity.

A comparison of kinetic parameters for a variety of nucleotide-sugars would be highly desirable in terms of understanding the mechanism that underlies the substrate specificity toward the donor. An investigation of the structural requirements of the donor nucleotide-sugar in terms of facilitating the transfer reaction would provide useful information relative to our understanding of the catalytic mechanism of action of GnT-III, because the structural elements of the transferred monosaccharide should be closely associated with the chemical basis of the reaction. In this study, we report on the preparation of a polyhistidine-tagged soluble form of rat GnT-III using a baculovirus–insect cell expression system and the kinetic characterization of the purified enzyme. Kinetic analyses using a variety of nucleotide-sugars as the donor substrate were carried out in order to elucidate the structural requirements of the transferred monosaccharides. The contribution of the nucleotide portion of the donor substrate was also investigated using a series of nucleotide-Glcs which contained different nucleotide moieties. Based on these experimental findings, we propose a possible mechanism for the donor substrate specificity of GnT-III, in which the donor is distinguished not *via* differences in binding affinity at the binding step but, rather, on the basis of catalysis.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and DNA modifying enzymes were purchased from Takara or New England Biolabs. Uridine 5′-diphosphosugars and various nucleotide-Glcs were obtained from Sigma. Oligonucleotide primers were synthesized by Greiner Japan. Other common chemicals were from Wako Pure Chemicals or Nacalai Tesque.

Site-Directed Mutagenesis—Site-directed mutagenesis was carried out according to Kunkel (*28*), as described previously (*29*) for the creation of a poly-histidine tag (His × 6) at the C-terminus of the GnT-III. The 3′ *Hin*dIII–*Bam*HI 0.44 kb fragment of rat GnT-III 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-His-His-His-His-His-His-Gly-Ala-Gly-(Stop). The oligonucleotide primer used in this study was 5′-GAT ACA ACG GAG GGC GGG GGC CAT CAC CAT CAC CAT CAC GGG GCC GGC TAG GAT GAT GGC TAA GCC-3′. The resulting mutation was verified by dideoxy sequencing using a DNA sequencer (Applied Biosystems, model 373A), as were the entire sequences which had been subjected to mutagenesis.

Construction of the Transfer Plasmid—A 5′ *Bsp*EI–*Eag*I 0.5 kb fragment from the open reading frame of the rat GnT-III cDNA (*25*) was ligated to *Xma*I and *Eag*I sites of a transfer vector, pAcGP67-c vector (PharMingen), which produces a protein that is fused to a cleavable signal peptide derived from baculoviral protein gp67. The 3′ *Hin*dIII–*Bam*HI fragment in which the tag sequence had been created as described above was subcloned with its upstream *Eag*I–*Hin*dIII 0.62 kb fragment into a pBluescript. The *Eag*I–*Bam*HI 1 kb fragment was excised from the resulting plasmid and inserted into the *Eag*I and *Bgl*II sites of the pAcGP67-c containing 5′ *Bsp*EI–*Eag*I fragment. This plasmid was used to express the fusion protein in which a GnT-III N-terminal sequence of about 60 residues was replaced by the gp67 signal peptide, and was subjected to a transfection experiment after purification using a QIAGEN plasmid mini kit.

Cell Culture and General Manipulation of Viruses—*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/liter yeastolate, 3.33 g/liter lactalbumin hydrolysate, and 100 mg/liter kanamycine. Recombinant viruses were manipulated as described (*30*).

Preparation of Recombinant Viruses—The purified transfer plasmid (1 μg) was co-transfected into 5 × 10^5 Sf21 cells with 10 ng of BaculoGold DNA (PharMingen), which was used as *Autographa californica* nuclear polyhedrosis viral genome. The transfection experiments were carried out by

the Lipofectin (GIBCO-BRL) method (*31*), as described previously (*32*, *33*). Medium containing the recombinant virus generated by homologous recombinations was collected 6 days after transfection. The recombinant virus was further amplified to more than 5×10^7 plaque forming units/ml prior to use.

Expression of Soluble Recombinant Rat GnT-III in Insect Cells—2×10^8 Sf21 cells were infected with the recombinant virus at a multiplicity of infection of more than 8. After incubation of the cells in the viral solution for 2 h, the medium was exchanged with serum-free medium, SFM900 (GIBCO-BRL). The medium was collected about 100 h post infection for purification of the secreted GnT-III.

Purification of the Recombinant Enzyme—One hundred milliliters of the medium was dialyzed against 20 mM sodium phosphate buffer, 0.1% Lubrol PX (pH 6.5). The dialyzed medium was then subjected to CM-Sepharose on a column pre-equilibrated with the same buffer as was used for the dialysis. The CM-Sepharose column was thoroughly washed with the buffer, and the bound proteins were eluted with 50 mM sodium phosphate buffer, 0.1% Lubrol PX, 500 mM NaCl (pH 6.5). Subsequently, the eluted fractions were applied to Ni^{2+}-chelating Sepharose, then washed thoroughly with 50 mM sodium phosphate buffer, 0.1% Lubrol PX, 500 mM NaCl, 80 mM imidazole (pH 6.5). The bound enzyme was eluted by raising the imidazole concentration in the buffer to 200 mM, then concentrated by Amicon YM-10. The purified enzyme was dialyzed against 20 mM sodium phosphate buffer, 0.1 mM EDTA, pH 6.5.

Electrophoresis—The purified enzymes were subjected to SDS-PAGE analysis on 10% gels, according to Laemmli (*34*), and the protein bands were visualized using a silver-staining kit (Daiichi Pure Chemicals).

Immunoblot Analysis—The purified protein was separated on SDS-PAGE, then electrophoretically transferred onto a PVDF membrane (Millipore). The membrane was blocked by treatment with 1% skim milk in PBS and reacted with anti GnT-III and anti-polyhistidine antibodies. After washing, the membrane was incubated with the appropriate second antibodies, which had been conjugated to horseradish peroxidase. After washing again, the reactive protein bands were visualized by chemiluminescence using an ECL kit (Amersham).

Standard Assay for Enzyme Activity and Kinetic Analysis—A standard assay for GnT-III activity was carried out using a fluorescent oligosaccharide substrate, as described previously (*35*, *36*). An agalacto biantennary sugar chain labeled with 2-aminopyridine (PA), GnGn-bi-PA, was used as the acceptor. The enzyme was incubated at 37°C with 5 μM GnGn-bi-PA and 20 mM UDP-GlcNAc as a donor substrate in 0.1 M MES-NaOH, 10 mM $MnCl_2$, 1% Triton X-100, 200 mM GlcNAc (pH 6.5). The reaction was terminated by boiling for 2 min, then the mixture was centrifuged at 15,000 rpm in a microcentrifuge for 10 min. The resulting supernatant was injected into a reversed phase HPLC apparatus equipped with a TSKgel ODS 80TM (4.6 × 150, TOSOH). The acceptor substrate and the product were separated isocratically using a 20 mM ammonium acetate buffer (pH 4.0) containing 0.3% *n*-butanol. The fluorescence of the eluate was monitored with a fluorescence detector (Shimazu, RF-10AXL) at excitation and emission wavelengths of 320 nm and 400 nm, respectively. For kinetic analyses, the purified recombinant GnT-III was incubated with various concentrations of GnGn-bi-PA and sugar nucleotides in 100 mM HEPES-NaOH buffer, 10 mM $MnCl_2$, pH 7.0. The products were analyzed by reversed phase HPLC under the same conditions as described for the standard assay.

Electrospray Ionization Mass Spectrometry (ESI-MS)—ESI-MS was carried out using an LCQ (Finnigan) quadrupole mass spectrometer equipped with an electrospray atmospheric pressure ionization source. The oligosaccharide-PA was dissolved in a 50% aqueous methanol solution containing 1% acetic acid and introduced into the ion source by a direct infusion at a flow rate of 3 μl/min using a syringe pump integrated into the 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 600–2000.

Sugar Composition Analysis—Component sugars were analyzed by the method of Hase *et al.* (*37*). The oligosaccharide-PA was hydrolyzed by incubation with 4 M trifluoroacetic acid at 100°C for 3 h. The resulting monosaccharides were subjected to re-*N*-acetylation by acetic anhydride, then pyridylaminated using a GlycoTAG (TAKARA) according to the manufacturer's instructions. After removal of excess reagents by use of a boronic acid–coupled polyacrylamide gel (Pierce), the PA-monosaccharides were analyzed by ion exchange HPLC on a TSKgel sugar AXI column (4.6 × 150, TOSOH). The PA-sugars were eluted isocratically with the solvent of 0.7 M boric acid–KOH (pH 9) and acetonitrile (9:1) at a flow rate of 0.3 ml/min. The column was heated at 73°C in a column oven. The eluted PA-sugars were monitored by means of a fluorescence detector at an excitation wavelength of 310 nm and an emission wave-

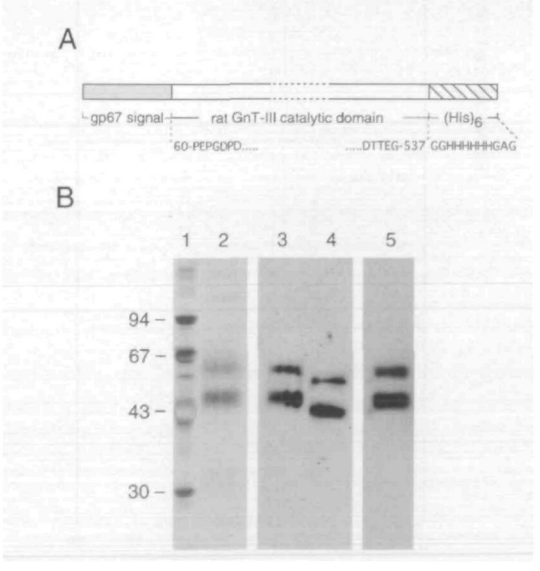

Fig. 1. **Expression of a soluble $(His)_6$-tagged form of GnT-III.** (A) A signal peptide derived from a baculoviral protein, gp67, and some additional residues were fused at the Pro-60 position of rat GnT-III. The fusion protein was also C-terminally tagged with a His × 6 sequence. (B) Proteins were separated on 10% SDS-gel and visualized by silver staining; lanes 1 and 2 indicate a molecular size marker and the purified recombinant GnT-III, respectively. GnT-III proteins were detected by immunoblot analysis using an anti–GnT-III antibody (lanes 3 and 4) or anti-$(His)_6$ antibody (lane 5). In lane 4, the protein was deglycosylated by PNGase-F.

length of 380 nm. These chromatographic conditions were also employed for the separation of a PA-sugar chain containing GalNAc.

Protein Determination—Protein contents were determined using a BCA protein assay kit (Pierce). Bovine serum albumin was used as a standard.

RESULTS

The recombinant rat GnT-III was expressed as a fusion protein with a signal peptide derived from a baculoviral protein, gp67, in order to permit the efficient secretion of the GnT-III into the medium. When the full-length GnT-III was expressed in a non-fused manner, a large amount of protein accumulated in the infected insect cells. However, since a major portion of the expressed protein appeared to be inactive and could not be solubilized with detergent, it was not possible to purify the active GnT-III from the cells. On the other hand, a significant level of activity was found in the culture medium of the infected insect cells, indicating that the secreted GnT-III was almost fully active. For a more efficient expression and secretion of the GnT-III, the N-terminal 60 amino acids were replaced by the viral protein signal sequence. Polyhistidine tag was also added to the C-terminus of the enzyme to permit purification *via* immobilized metal affinity chromatography, as shown in Fig. 1A.

The recombinant GnT-III, which was expressed in a soluble and polyhistidine-tagged form, was purified from the culture medium. The specific activity of the purified rat GnT-III was determined to be 1.2 μmol/h/mg under stan-

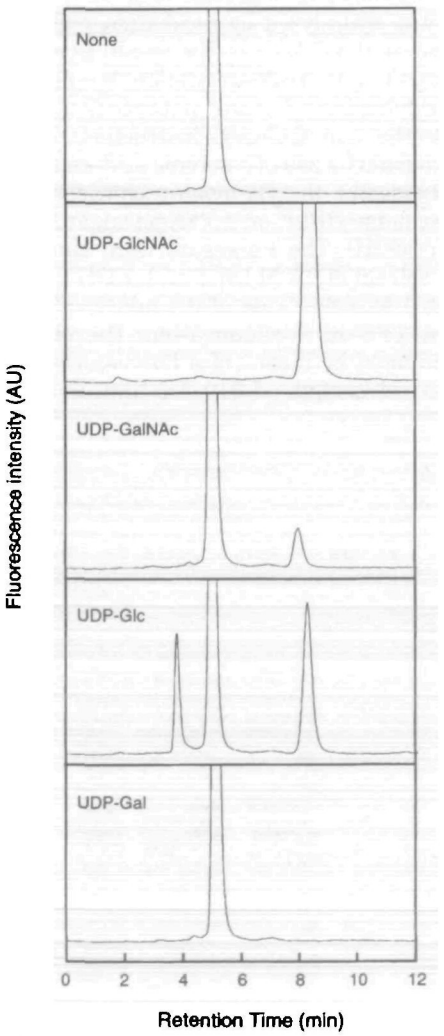

Fig. 2. **Detection of GnT-III activities using various UDP-sugars.** The PA-labeled agalacto biantennary sugar chain was incubated with purified GnT-III in the absence or presence of the indicated UDP-sugars. The peak at an elution time of 4.9 min represents unreacted substrate. The peak at 8.2 min in the reaction with UDP-Glc represents a product in which GlcNAc was transferred using a trace amount of UDP-GlcNAc contaminant in UDP-Glc. Details of the reaction conditions and chromatographic procedures are described in the text.

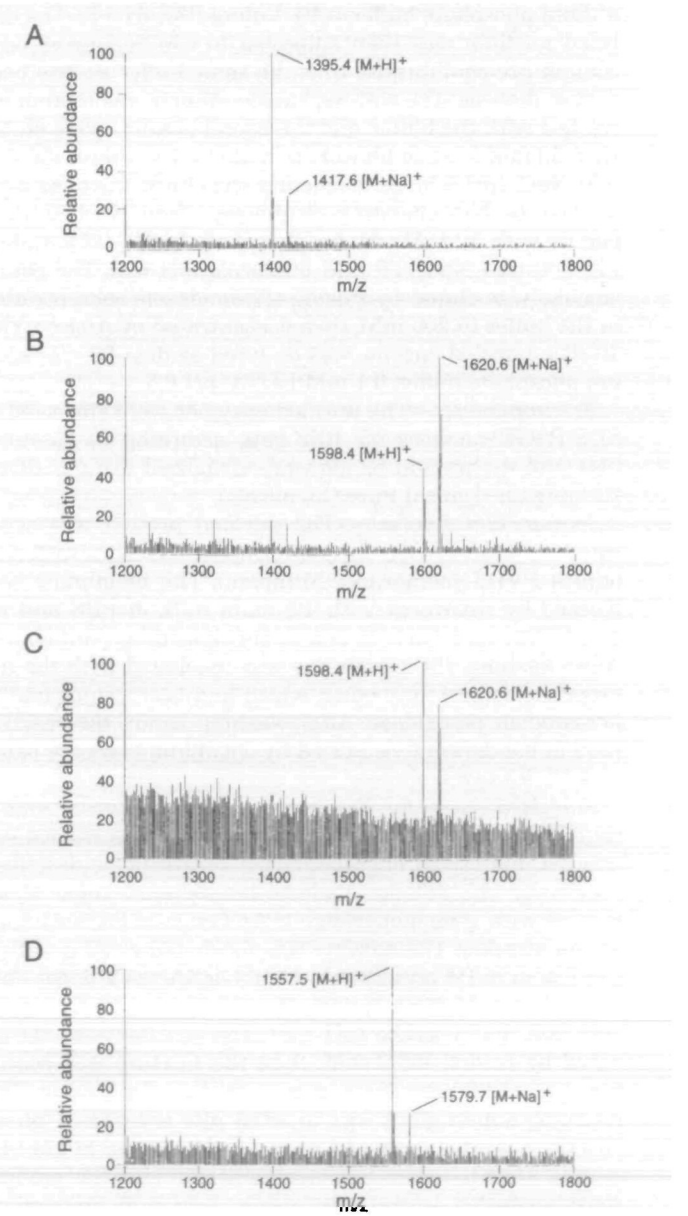

Fig. 3. **ESI-MS analysis of the oligosaccharide products from the reactions with UDP-sugars.** MS spectra of the products from the reactions with UDP-GlcNAc (B), UDP-GalNAc (C), and UDP-Glc (D) are shown with the spectrum of the substrate oligosaccharide (A). Conditions are detailed under "EXPERIMENTAL PROCEDURES."

dard assay conditions in which 10 mM UDP-GlcNAc and 5 μM agalacto biantennary-PA were used as the donor and acceptor substrates, respectively. Thirty to 40 μg of the purified enzyme was obtained from 100 ml of the culture medium, in a yield of approximately 20% and a 200-fold purification. SDS-PAGE analysis of the purified protein indicated two protein bands having molecular masses of 60 and 50 kDa, both of which were found to be the GnT-III protein with an intact C-terminal His × 6 sequence, as indicated by immunoblot analysis using a GnT-III–specific antibody and an anti-polyhistidine antibody (Fig. 1B). In addition, PNGase-F digestion revealed that both species were glycosylated to a similar extent. These findings indicated that the difference in molecular mass was due to the different proteolytic cleavage sites in the stem region.

To explore the substrate specificity toward donor nucleotide-sugars, enzyme activities were assessed using various UDP-sugars. The purified enzyme was incubated with 2 μM acceptor and 2 mM UDP-sugars for 10 h, and the products were analyzed by reversed phase HPLC (Fig. 2). In the chromatographic separation, the agalacto biantennary-PA used as the acceptor substrate was eluted at 4.9 min, whereas the peak for the corresponding usual bisected sugar chain, to which GlcNAc had been transferred, was found at 8.2 min. In the incubation with a natural donor, UDP-GlcNAc, no unreacted substrate remained, due to the much faster reaction rate. On the other hand, when the enzyme was incubated with UDP-GalNAc and UDP-Glc, the reactions gave products which had retention times of about 8.0 min and 3.8 min, respectively. However, no product was found as the result of incubation with UDP-Gal under these conditions.

To confirm the transfers of GalNAc and Glc by GnT-III, the reaction products were collected and analyzed by ESI-MS. As shown in Fig. 3, mass spectrometric analyses showed that the m/z values for $(M+H)^+$ were 1,395.4 for the substrate agalacto biantennary, 1,598.4 for the bisected oligosaccharide to which GlcNAc had been transferred, 1,598.4 for the GalNAc-transferred product, and 1,557.5 for the Glc-transferred product. Thus, it was found that the product obtained from the reaction with UDP-Glc contains 1 mol of Glc, suggesting that the recombinant GnT-III is capable of transferring Glc from UDP-Glc to the acceptor oligosaccharide. The product of the transfer of GalNAc gave the same m/z as the GlcNAc-transferred product, the bisected oligosaccharide-PA, as expected, and, in addition, the GalNAc-transferred product was not separated from the GlcNAc-product by the reversed phase HPLC. Thus, in order to further confirm the transfer of GalNAc, the product oligosaccharide from the reaction with UDP-GalNAc was subjected to component sugar analysis and anion exchange chromatography using a borate buffer (Fig. 4, A and B). The component sugar analysis showed that the product contained GalNAc, and that this product was evidently separated from the usual GlcNAc-transferred bisected sugar chain, probably because of the *cis*-diol of the GalNAc residue in the anion exchange chromatography. In addition, the product obtained from the reaction with UDP-GalNAc contained no detectable GlcNAc-transferred product.

When the enzyme activity in the culture medium and the purified enzyme was retrospectively determined using UDP-GalNAc and UDP-Glc as well as UDP-GlcNAc, it was found that the ratio of the specific activities of the purified enzyme to those in the culture medium was the same for all these donors, indicating that the activities with respect to UDP-GalNAc and UDP-Glc were perfectly co-purified

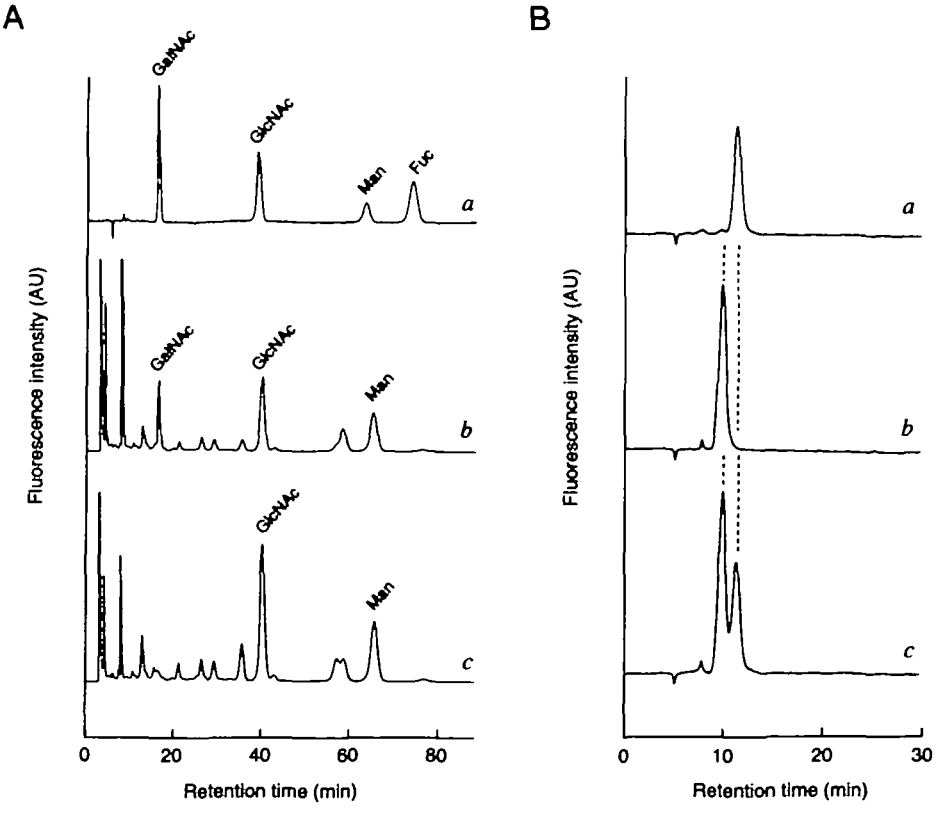

Fig. 4. **Identification of GalNAc transfer by GnT-III.** (A) Component monosaccharide analysis. TFA-hydrolyzed products were pyridylaminated and separated by ion-exchange HPLC. Traces a–c indicate standard pyridylaminated monosaccharides, the product from the reaction with UDP-GalNAc and GlcNAc-transferred product, respectively. Conditions are detailed under "EXPERIMENTAL PROCEDURES." (B) Separation of the GalNAc-transferred product from the GlcNAc-transferred one. Traces a and b indicate the GalNAc- and GlcNAc-products, respectively. In trace c, a mixture of both two products was injected into the HPLC.

with the usual GlcNAc-transferring activity. Furthermore, enzyme activities with respect to transferring GalNAc and Glc to the acceptor substrate were not detected in the culture medium of the Sf21 cells which were non-infected or infected with the recombinant baculovirus, which contained a cDNA for an unrelated glycosyltransferase, α1,6fucosyltransferase (*38*). Therefore, it could be clearly demonstrated that the GnT-III is able to transfer GalNAc and Glc from the corresponding UDP-sugars, albeit at much slower reaction rates than that of GlcNAc. Reaction rates, which are based on the initial velocity under the same conditions, were found to be 0.1% and 0.2% for UDP-GalNAc and UDP-Glc, respectively, compared to the rate for UDP-GlcNAc.

The elucidation of a kinetic factor associated with the distinct transfer rates on which the donor substrate specificity of GnT-III may be based would be important in understanding the mechanism underlying the specificity. To explore the kinetic basis of the donor substrate specificity of GnT-III, kinetic analyses of reactions involving UDP-GalNAc and UDP-Glc as well as UDP-GlcNAc were carried out. As shown in Fig. 5, the reciprocal plots obtained from

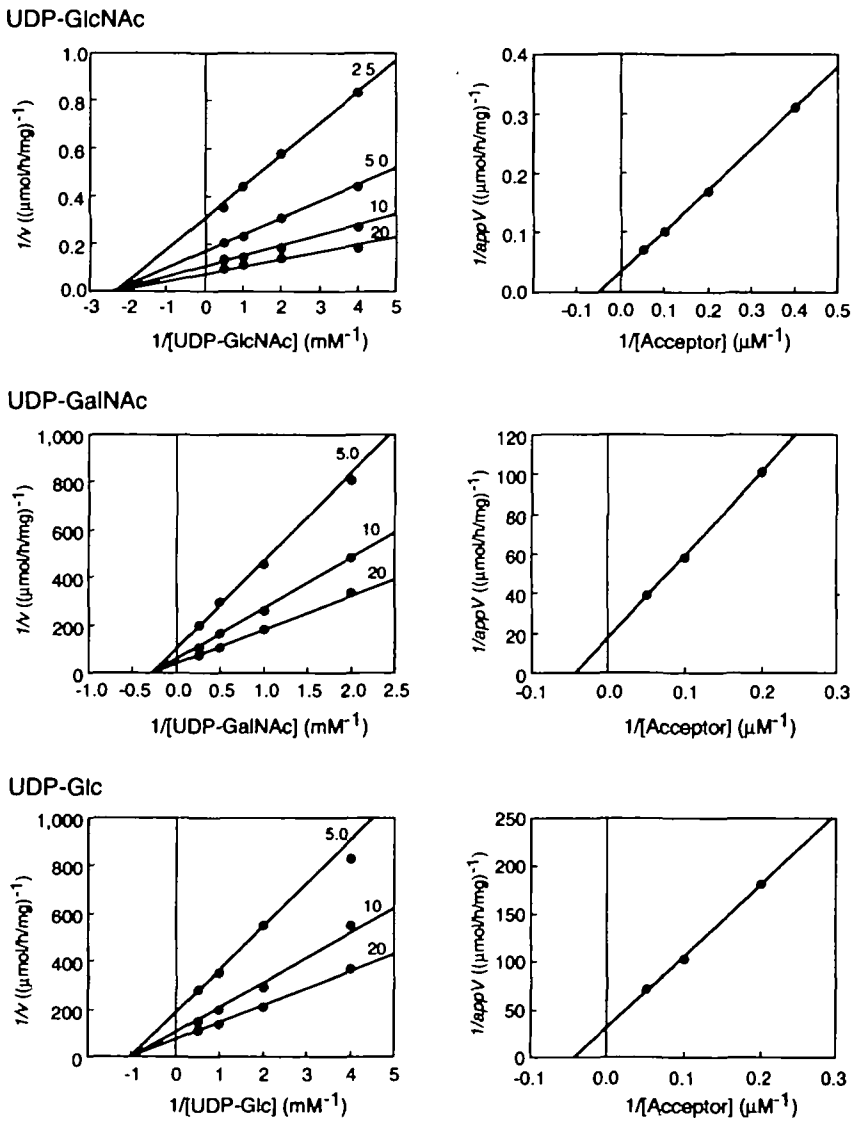

Fig. 5. **Kinetic analysis of the GnT-III reactions involving UDP-GlcNAc, UDP-GalNAc, and UDP-Glc.** Numbers beside the plot in the left panels indicate acceptor concentrations. The right panels are secondary plots of $1/appV_{max}$ as a function of reciprocal values of acceptor concentrations.

TABLE I. **Kinetic parameters for the transfer reactions with UDP-GlcNAc, UDP-GalNAc, and UDP-Glc by GnT-III.**

Donor	V_{max}		Donor			Acceptor[a]
			K_m	k_{cat}/K_m		K_m
	(μmol/h/mg)	(%)	(mM)	($M^{-1}s^{-1}$)	(%)	(μM)
UDP-GlcNAc	30	(100)	0.42	990	(100)	21
UDP-GalNAc	0.058	(0.19)	3.6	0.22	(0.022)	24
UDP-Glc	0.031	(0.10)	1.0	0.43	(0.043)	23

[a]Agalacto biantennary oligosaccharide-PA was used as an acceptor substrate.

assays with various concentrations of donors and the acceptor indicated that plot sets for various concentrations of the acceptor intersected the X-axis at the same point, suggesting that the reaction mechanism for GnT-III is consistent with a sequential mechanism, probably a rapid equilibrium random mechanism. The K_m values for the donor and acceptor were mutually independent of the concentration of the other substrate. These kinetic properties of GnT-III were confirmed for all cases of donor UDP-sugars examined.

Table I summarizes the kinetic parameters for the reactions with various donor substrates. While the K_m values for the acceptor, which were determined using various donors, were essentially the same, the K_m values for the donors were slightly different. The K_m values for UDP-GalNAc and UDP-Glc were only 8.6 and 2.4 times, respectively, higher than the value for UDP-GlcNAc. This suggests that the respective structural differences with respect to the monosaccharides from GlcNAc, *i.e.*, the configuration of the 4-hydroxyl group and the presence of the 2-*N*-acetyl group, do not significantly affect the binding of the donor in a manner that would account for the distinct reaction rates. On the other hand, the V_{max} values found in analyses using UDP-GalNAc and UDP-Glc as the donor are 530 and 970 times lower, respectively, than that for UDP-GlcNAc. These much lower V_{max} values account for the much lower rates of transfers of GalNAc and Glc, and suggest that the specificity toward the donor is largely dependent on a catalytic step, rather than the actual binding of the substrate. On the basis of the catalytic efficiency (k_{cat}/K_m for the donor), the specificity of GnT-III with respect to UDP-GlcNAc was estimated to be at least 2,000 times higher than that of the other two UDP-sugars examined.

The kinetic analyses indicate that the binding of the donor is not dependent on the monosaccharide portion of the donor substrate, since the replacement of the GlcNAc moiety by GalNAc and Glc did not significantly affect the K_m values. The inhibition of GnT-III activity by GlcNAc, α-GlcNAc-1-phosphate and UDP also provides support for this conclusion. Consistent with the results from the kinetic analyses, only UDP markedly inhibited the enzyme activity whereas the monosaccharide and its 1-phosphate derivative had only a negligible effect, as shown in Fig. 6. These findings are consistent with the donor binding to the enzyme *via* the interaction of its nucleotide portion with the enzyme, as is believed to occur for many glycosyltransferases (*38–40*). Other diphosphonucleotides, ADP, CDP, TDP, and GDP, also inhibited the activity of the enzyme, probably by mimicking UDP (data not shown).

For a more detailed investigation of the involvement of the nucleotide portion of the donor in the reaction, we examined whether nucleotide-sugars with a nucleotide portion that differs from UDP could serve as a donor for GnT-III. Since UDP-Glc is able to serve as a donor substrate for GnT-III, an investigation using a variety of nucleotide-Glcs would allow the contribution of a nucleotide moiety to the transfer reaction by this enzyme to be evaluated. As shown in Fig. 7, the activity assay indicated that the incubation of GnT-III with ADP-, CDP-, TDP-, and GDP-Glc yielded no product, whereas Glc was transferred from UDP-Glc, suggesting an absolute requirement for uridine diphosphate in the GnT-III reaction. However, when these inactive nucleotide-Glc derivatives were added to a reaction in which UDP-GlcNAc was used as the donor, the transfer of GlcNAc was inhibited. Moreover, it was also found that ADP-, CDP,

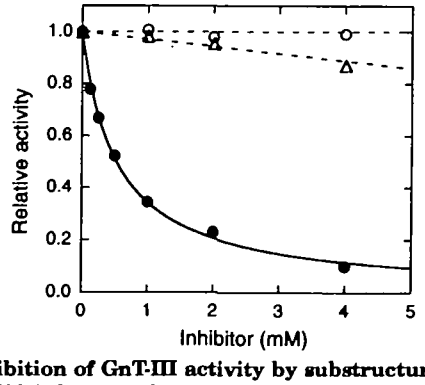

Fig. 6. **Inhibition of GnT-III activity by substructural components of UDP-GlcNAc.** Closed circles denote UDP, open circles GlcNAc, and open triangles α-GlcNAc-1-phosphate. The activity was assayed in the presence of 1 mM UDP-GlcNAc and 5 μM acceptor.

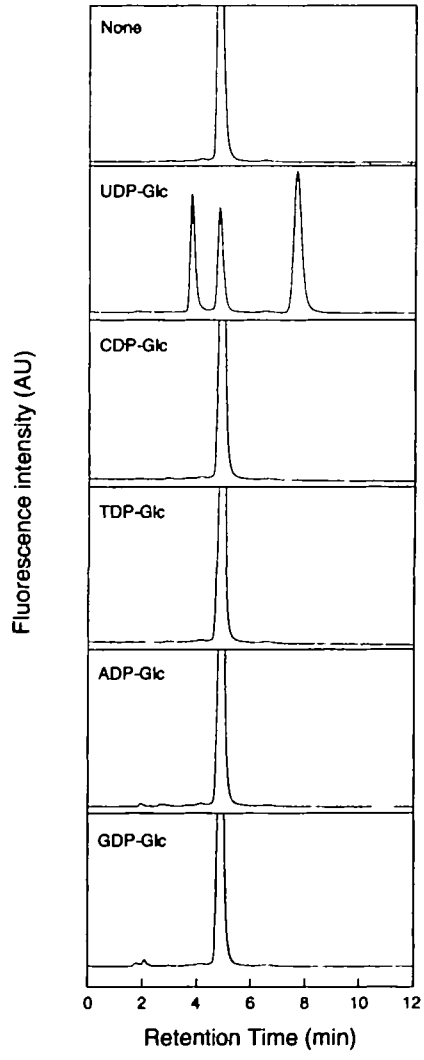

Fig. 7. **Reactions with various nucleotide-Glc derivatives.** The purified GnT-III was incubated without or with various nucleotide-Glc derivatives as a donor in the presence of acceptor. Concentrations of the nucleotide-Glc were 2 mM.

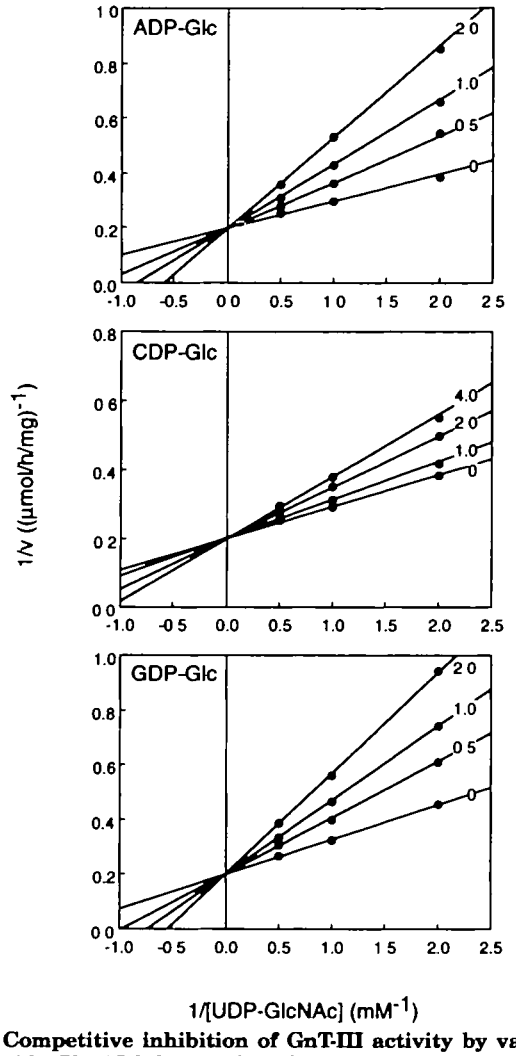

Fig. 8. Competitive inhibition of GnT-III activity by various nucleotide-Glc. ADP-Glc and GDP-Glc were added to the reaction at 0, 0.5, 1.0, 2.0 mM. Concentrations of CDP-Glc were 0, 1.0, 2.0, 4.0 mM. Acceptor substrate concentration was used at 5 μM.

and GDP-Glc inhibit the enzyme in a competitive manner against UDP-GlcNAc (Fig. 8), indicating that these nucleotide-Glc derivatives bind to the subsite for the donor in spite of their inability to serve as a donor substrate. Although the enzyme was also inhibited by TDP-Glc, this inhibition could not be classified. It might have been a more complicated inhibition involving multiple inhibition mechanisms. The K_i values in the competitive inhibition by those nucleotide-Glc derivatives, which were obtained from the secondary plots, were comparable to the K_m for UDP-Glc (Table II). This suggests that GnT-III binds various nucleotide-sugars at the active site to form the complex without discriminating between the different nucleotide portions. However, it seems likely that the chemical process exclusively involves UDP as the nucleotide moiety of the donor substrate.

DISCUSSION

In this study, the purified recombinant GnT-III was kinetically characterized, and the kinetic basis for the donor substrate specificity was investigated by the use of a variety of UDP-sugars and other nucleotide-sugar derivatives. Our findings show that the enzyme binds various nucleotide-sugars in the subsite for the donor with comparable affinities to that for UDP-GlcNAc, as indicated by comparison of the K_m values from the kinetic analyses and the K_i values for competitive inhibition against the natural donor. However, in the case of the enzyme-nucleotide-sugar complex with the acceptor, the transfer of the monosaccharide from the donor appears to be executed highly specifically for the appropriate combination of nucleotide and monosaccharide, namely, a combination of UDP and GlcNAc. In contrast to many other enzymes whose substrate specificity is believed to depend to a large extent on the formation of an enzyme-substrate complex, the substrate specificity of GnT-III toward the donor does not appear to be conferred by the formation of the enzyme-substrate complex which results from the specific binding. It is more likely that the underlying mechanism for donor specificity involves the subsequent catalytic process, which appears to be dependent on the structural elements of the monosaccharide moiety, such as the configuration at the 4-hydroxyl group and the presence of a 2-*N*-acetyl group. It was also shown that uridine plays an essential role in the chemical step but not in donor binding. These results are consistent with the conclusion that the substrate specificity of GnT-III toward the donor is determined on a catalytic basis, rather than by specific binding, in terms of distinguishing UDP-GlcNAc from various structurally similar nucleotide-sugars that serve as the donors for many other glycosyltransferases.

TABLE II. **K_i values of various nucleotide-Glcs in the competitive inhibition against UDP-GlcNAc.**

Nucleotide-Glc	K_i (mM)
UDP-Glc	(1.0)[a]
TDP-Glc	n.d.[b]
ADP-Glc	0.75
CDP-Glc	3.9
GDP-Glc	1.0

[a] K_m value. [b] K_i value in the competitive inhibition was not determined because the inhibition by TDP-Glc could not be classified.

Differences in the kinetic parameters between UDP-GlcNAc and UDP-GalNAc may be ascribed only to the effect of the different configurations of the hydroxyl group at the C-4 position. The K_m for the donor was only modestly increased in the case of an axial hydroxyl group at the C-4 position (GalNAc) in the donor monosaccharide, as compared to that of an equatorial hydroxyl (GlcNAc), suggesting that the contribution of the 4-hydroxyl to the binding of the nucleotide-sugar to the enzyme is not significant. Regarding the K_m as a dissociation constant, the difference in ΔG for the binding of the donor between UDP-GlcNAc and UDP-GalNAc was calculated to be only 1.3 kcal/mol. On the other hand, the replacement of the equatorial hydroxyl by an axial one at the C-4 position led to a large decrease in the value of V_{max}, suggesting the importance of an equatorial hydroxyl group in the catalysis (Table I). Calculating from this decrease in V_{max} or k_{cat}, the destabilization of the transition state as the result of altering the configuration at C-4 was estimated to be 3.9 kcal/mol. It therefore appears that the 4-hydroxyl group plays a more critical role in the catalysis than in the prior binding step for the chemical process. It is possible that this value for

destabilization might correspond to the loss of a critical hydrogen bond involving the equatorial hydroxyl group at C-4 position, which may be formed in the transition state. Such a hydrogen bond could facilitate the conformational change of the donor monosaccharide, which would be expected to occur in conjunction with the inversion of the anomeric center during the transition state, thereby contributing to the catalysis. Since the absence of a 2-*N*-acetyl group in the donor affects the kinetic parameters, which are based on the difference in the parameters between UDP-GlcNAc and UDP-Glc, in a similar way to the alteration of the C-4 configuration, the 2-*N*-acetyl group appears to play a similar role to the equatorial C-4 hydroxyl group in the reaction by GnT-III. In this case, while the replacement of the 2-*N*-acetyl group by a hydroxyl group caused a destabilization of only 0.53 kcal/mol on binding, the increase in the activation energy or the destabilization of the transition state was estimated to be as large as 4.3 kcal/mol. Considering the additive effects of both structural factors on the kinetic parameters, it seems reasonable that UDP-Gal could actually no longer act as the donor. Destabilization of the activation energy would be expected to be 8.2 kcal/mol, which corresponds to a value of 2.0×10^{-6} for the V_{max}, compared to that for UDP-GlcNAc.

The present study has also shown that GnT-III has an absolute requirement for UDP as the nucleotide portion of the donor in the transfer of monosaccharide, even though the enzyme accommodates the binding of various nucleotide-Glcs to the subsite for the donor in the active site. Thus, it is likely that GnT-III "non-specifically" binds a nucleotide-sugar at the donor site *via* the interaction with its nucleotide moiety, even if it is not UDP, but the enzyme specifically transfers a monosaccharide only from the UDP-sugar derivatives. Although this suggests that the UDP portion participates in the catalytic process during the reaction catalyzed by GnT-III, the mechanism by which UDP facilitates the transfer of the monosaccharide is not presently clear. Some glycosyltransferases display an obvious preference for the specific nucleotide of a donor in binding (*40*), while others tolerate a variety of nucleotide moieties in transferring the monosaccharide (*41*). Since even a TDP-sugar, which differs from the corresponding UDP-sugar only by virtue of a substitution of a 5-methyl group in the pyrimidine ring, failed to serve as the donor in the reaction catalyzed by GnT-III. The structural requirement for the nucleotide base seems to be nearly absolute for this enzyme, in terms of catalysis.

The glycosyltransferases involved in the biosyntheses of blood groups A and B are very homologous, and only four amino acid residues differ between these two enzymes (*42*, *43*). The A and B glycosyltransferases transfer GalNAc and Gal from their UDP-conjugates, respectively. It has been reported that these substrate specificities can be interconverted by a single amino acid substitution (*44*). Kinetic analyses have shown that the alteration of the substrate specificity is controlled by both the K_m value and k_{cat} (*45*, *46*), which is consistent with the proposal that the nucleotide-sugar donor specificity of glycosyltransferases is also controlled by the catalytic process.

Substantial dependence of the catalytic process on the monosaccharide structural elements (and, in some cases, a nucleotide portion), the combination of which is unique to the particular nucleotide-sugar that is utilized as the donor, would contribute to an ability to distinguish the donor from various similar nucleotide-sugars which coexist in the Golgi apparatus and which are available as substrates for other glycosyltransferases. If the substrate specificity of glycosyltransferases were determined only by the specific binding, as reflected by differences in K_m values, the strict regulation of oligosaccharide construction would greatly be impaired; in the absence or presence of a considerably low concentration of the "true" donor, one glycosyltransferase might transfer a different sugar at a significant rate, using a different nucleotide-sugar whose concentration is sufficiently high. In contrast, if the specificity were based on the catalytic process, the reaction rate for different donors would remain very slow under the same conditions, leading to the suppression of an unfavorable transfer that might disturb or affect the subsequent reaction by another glycosyltransferase. Such a donor substrate specificity of one glycosyltransferase would be relatively insensitive to variation of the concentrations of the true donor and other nucleotide-sugars that might interfere with the action of the enzyme. Therefore, this type of substrate specificity would be preferred for a properly ordered, stepwise addition of monosaccharides in oligosaccharide biosyntheses. We therefore emphasize that the donor substrate specificity which is determined on the basis of catalysis constitutes an important factor in the function of glycosyltransferases.

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REFERENCES

1. Narasimhan, S. (1982) Control of glycoprotein synthesis. UDP-GlcNAc:glycopeptide β4-N-acetylglucosaminyltransferase III, an enzyme in hen oviduct which adds GlcNAc in β1-4 linkage to the beta-linked mannose of the trimannosyl core of N-glycosyl oligosaccharides. *J. Biol. Chem.* **257**, 10235–10242
2. Schachter, H., Narasimhan, S., Gleeson, P., and Vella, G. (1983) Control of branching during the biosynthesis of asparagine-linked oligosaccharides. *Can. J. Biochem. Cell Biol.* **61**, 1049–1066
3. Bendiak, B. and Schachter, H. (1987) Control of glycoprotein synthesis. Kinetic mechanism, substrate specificity, and inhibition characteristics of UDP-N-acetylglucosamine:α-D-mannoside β1-2 N-acetylglucosaminyltransferase II from rat liver. *J. Biol. Chem.* **262**, 5784–5790
4. Allen, S.D., Tsai, D., and Schachter, H. (1984) Control of glycoprotein synthesis. The in vitro synthesis by hen oviduct membrane preparations of hybrid asparagine-linked oligosaccharides containing 5 mannose residues. *J. Biol. Chem.* **259**, 6984–6990
5. Gleeson, P.A. and Schachter, H. (1983) Control of glycoprotein synthesis. *J. Biol. Chem.* **258**, 6162–6173
6. 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
7. Easton, E.W., Bolscher, J.G., and van den Eijnden, D.H. (1991) Enzymatic amplification involving glycosyltransferases forms the basis for the increased size of asparagine-linked glycans at the surface of NIH 3T3 cells expressing the N-ras proto-oncogene. *J. Biol. Chem.* **266**, 21674–21680
8. Miyoshi, E., Ihara, Y., Hayashi, N., Fusamoto, H., Kamada, T., and Taniguchi, N. (1995) Transfection of N-acetylglucosaminyltransferase III gene suppresses expression of hepatitis B virus in a human hepatoma cell line, HB611. *J. Biol. Chem.* **270**, 28311–28315

9. 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
10. Yoshimura, M., Ihara, Y., Matsuzawa, Y., and Taniguchi, N. (1996) Aberrant glycosylation of E-cadherin enhances cell-cell binding to suppress metastasis. *J. Biol. Chem.* **271**, 13811–13815
11. Yoshimura, M., Ihara, Y., Ohnishi, A., Ijuhin, N., Nishiura, T., Kanakura, Y., Matsuzawa, Y., and Taniguchi, N. (1996) Bisecting N-acetylglucosamine on K562 cells suppresses natural killer cytotoxicity and promotes spleen colonization. *Cancer Res.* **56**, 412–418
12. Sheng, Y., Yoshimura, M., Inoue, S., Oritani, K., Nishiura, T., Yoshida, H., Ogawa, M., Okajima, Y., Matsuzawa, Y., and Taniguchi, N. (1997) Remodeling of glycoconjugates on CD44 enhances cell adhesion to hyaluronate, tumor growth and metastasis in B16 melanoma cells expressing β1,4-N-acetylglucosaminyltransferase III. *Int. J. Cancer* **73**, 850–858
13. Ihara, Y., Sakamoto, Y., Mihara, M., Shimizu, K., and Taniguchi, N. (1997) Overexpression of N-acetylglucosaminyltransferase III disrupts the tyrosine phosphorylation of Trk with resultant signaling dysfunction in PC12 cells treated with nerve growth factor. *J. Biol. Chem.* **272**, 9629–9634
14. Sultan, A.S., Miyoshi, E., Ihara, Y., Nishikawa, A., Tsukada, Y., and Taniguchi, N. (1997) Bisecting GlcNAc structures act as negative sorting signals for cell surface glycoproteins in forskolin-treated rat hepatoma cells. *J. Biol. Chem.* **272**, 2866–2872
15. Rebbaa, A., Yamamoto, H., Saito, T., Meuillet, E., Kim, P., Kersey, D.S., Bremer, E.G., Taniguchi, N., and Moskal, J.R. (1997) Gene transfection-mediated overexpression of β1,4-N-acetylglucosamine bisecting oligosaccharides in glioma cell line U373 MG inhibits epidermal growth factor receptor function. *J. Biol. Chem.* **272**, 9275–9279
16. Ihara, Y., Yoshimura, M., Miyoshi, E., Nishikawa, A., Sultan, A.S., Toyosawa, S., Ohnishi, A., Suzuki, M., Yamamura, K., Ijuhin, N., and Taniguchi, N. (1998) Ectopic expression of N-acetylglucosaminyltransferase III in transgenic hepatocytes disrupts apolipoprotein B secretion and induces aberrant cellular morphology with lipid storage. *Proc. Natl. Acad. Sci. USA* **95**, 2526–2530
17. Narasimhan, S., Schachter, H., and Rajalakshmi, S. (1988) Expression of N-acetylglucosaminyltransferase III in hepatic nodules during rat liver carcinogenesis promoted by orotic acid. *J. Biol. Chem.* **263**, 1273–1281
18. Nishikawa, A., Fujii, S., Sugiyama, T., Hayashi, N., and Taniguchi, N. (1988) High expression of an N-acetylglucosaminyltransferase III in 3′-methyl DAB-induced hepatoma and ascites hepatoma. *Biochem. Biophys. Res. Commun.* **152**, 107–112
19. Pascale, R., Narasimhan, S., and Rajalakshmi, S. (1989) Expression of N-acetylglucosaminyltransferase III in hepatic nodules generated by different models of rat liver carcinogenesis. *Carcinogenesis* **10**, 961–964
20. Ishibashi, K., Nishikawa, A., Hayashi, N., Kasahara, A., Sato, N., Fujii, S., Kamada, T., and Taniguchi, N. (1989) N-acetylglucosaminyltransferase III in human serum, and liver and hepatoma tissues: increased activity in liver cirrhosis and hepatoma patients. *Clin. Chim. Acta* **185**, 325–332
21. Nishikawa, A., Gu, J., Fujii, S., and Taniguchi, N. (1990) Determination of N-acetylglucosaminyltransferases III, IV and V in normal and hepatoma tissues of rats. *Biochim. Biophys. Acta* **1035**, 313–318
22. Miyoshi, E., Nishikawa, A., Ihara, Y., Gu, J., Sugiyama, T., Hayashi, N., Fusamoto, H., Kamada, T., and Taniguchi, N. (1993) N-acetylglucosaminyltransferase III and V messenger RNA levels in LEC rats during hepatocarcinogenesis. *Cancer Res.* **53**, 3899–3902
23. Yamashita, K., Koide, N., Endo, T., Iwaki, Y., and Kobata, A. (1989) Altered glycosylation of serum transferrin of patients with hepatocellular carcinoma. *J. Biol. Chem.* **264**, 2415–2423
24. Yamashita, K., Hitoi, A., Taniguchi, N., Yokosawa, N., Tsukada, Y., and Kobata, A. (1983) Comparative study of the sugar chains of γ-glutamyltranspeptidases purified from rat liver and rat AH-66 hepatoma cells. *Cancer Res.* **43**, 5059–5063
25. 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-1,4N-acetylglucosaminyltransferase III from rat kidney. *J. Biol. Chem.* **267**, 18199–18204
26. 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.* **113**, 692–698
27. Taniguchi, N. and Ihara, Y. (1995) Recent progress in the molecular biology of the cloned N-acetylglucosaminyltransferases. *Glycoconj. J.* **12**, 733–738
28. Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**, 488–492
29. Ikeda, Y., Fujii, J., Taniguchi, N., and Meister, A. (1995) Human γ-glutamyl transpeptidase mutants involving conserved aspartate residues and the unique cysteine residue of the light subunit. *J. Biol. Chem.* **270**, 12471–12475
30. Piwnica-Worms, H. (1987) *Current Protocols in Molecular Biology* (Ausubel, F.M., Brent, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds.) pp. 16.8.1–16.11.7, Greene Publishing Associates and Wiley-Interscience, New York
31. Felgner, P.L., Gadek, T.R., Holm, H., Roman, R., Chan, H.W., Wenz, M., Northrop, J.R., Ringold, G.M., and Danielsen, M. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**, 7413–7417
32. Ikeda, Y., Fujii, J., Taniguchi, N., and Meister, A. (1995) Expression of an active glycosylated human γ-glutamyl transpeptidase mutant that lacks a membrane anchor domain. *Proc. Natl. Acad. Sci. USA* **92**, 126–130
33. Ikeda, Y., Fujii, J., Anderson, M.E., Taniguchi, N., and Meister, A. (1995) Involvement of Ser-451 and Ser-452 in the catalysis of human γ-glutamyl transpeptidase. *J. Biol. Chem.* **270**, 22223–22228
34. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
35. Nishikawa, A., Fujii, S., Sugiyama, T., and Taniguchi, N. (1988) A method for the determination of N-acetylglucosaminyltransferase III activity in rat tissues involving HPLC. *Anal. Biochem.* **170**, 349–354
36. Taniguchi, N., Nishikawa, A., Fujii, S., and Gu, J.G. (1989) Glycosyltransferase assays using pyridylaminated acceptors:N-acetylglucosaminyltransferase III, IV, and V. *Methods Enzymol.* **179**, 397–408
37. Hase, S., Hatanaka, K., Ochiai, K., and Shimizu, H. (1992) Improved method for the component sugar analysis of glycoproteins by pyridylamino sugars purified with immobilized boronic acid. *Biosci. Biotechnol. Biochem.* **56**, 1676–1677
38. Takahashi, T., Ikeda, Y., Tateishi, A., Yamaguchi, Y., Ishikawa, M., and Taniguchi, N. (2000) A sequence motif involved in the donor substrate binding by α1,6-fucosyltransferase: the role of the conserved arginine residues. *Glycobiology* **10**, 503–510
39. Murray, B.W., Takayama, S., Schultz, J., and Wong, C.-H. (1996) Mechanism and specificity of human α-1,3-fucosyltransferase V. *Biochemistry* **35**, 11183–11195
40. Elhammer, A.P., Kezdy, F.J., and Kurosaka, A. (1999) The acceptor specificity of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases. *Glycoconj. J.* **16**, 171–180
41. Paul, P., Kamisaka, Y., Marks, D.L., and Pagano, R.E. (1996) Purification and characterization of UDP-glucose:ceramide glucosyltransferase from rat liver Golgi membranes. *J. Biol. Chem.* **271**, 2287–2293
42. Yamamoto, F., Marken, J., Tsuji, T., White, T., Clausen, H., and Hakomori, S. (1990) Cloning and characterization of DNA complementary to human UDP-GalNAc:Fucα1-2Gal α1-3GalNAc transferase (histo-blood group A transferase) mRNA. *J. Biol. Chem.* **265**, 1146–1151

43. Yamamoto, F., Clausen, H., White, T., Marken, J., and Hakomori, S. (1990) Molecular genetic basis of the histo-blood group ABO system. *Nature* **345**, 229–233
44. Yamamoto, F. and McNeill, P.D. (1996) Amino acid residue at codon 268 determines both activity and nucleotide-sugar donor substrate specificity of human histo-blood group A and B transferases. In vitro mutagenesis study. *J. Biol. Chem.* **271**, 10515–10520
45. Seto, N.O.L., Palcic, M.M., Compston, C.A., Li, H., Bundle, D.R., and Narang, S.A. (1997) Sequential interchange of four amino acids from blood group B to blood group A glycosyltransferase boosts catalytic activity and progressively modifies substrate recognition in human recombinant enzymes. *J. Biol. Chem.* **272**, 14133–14138
46. Seto, N.O.L., Compston, C.A., Evans, S.V., Bundle, D.R., Narang, S.A., and Palcic, M.M. (1999) Donor substrate specificity of recombinant human blood group A, B and hybrid A/B glycosyltransferases expressed in *Escherichia coli*. *Eur. J. Biochem.* **259**, 770–775