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Kinetic properties and substrate specificities of two recombinant human *N*-acetylglucosaminyltransferase-IV isozymes

Suguru Oguri · Aruto Yoshida · Mari T. Minowa · Makoto Takeuchi

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Abstract *N*-acetylglucosaminyltransferase (GnT)-IV catalyzes the formation of the GlcNAcβ1-4 branch on the GlcNAcβ1-2Manα1-3 arm of the core structure of *N*-glycans. Two human GnT-IV isozymes (GnT-IVa and GnT-IVb) had been identified, which exhibit different expression profiles among human tissues and cancer cell lines. To clarify the enzymatic properties of the respective enzymes, their kinetic parameters were determined using recombinant full-length enzymes expressed in COS7 cells. The K_m of human GnT-IVb for UDP-GlcNAc was estimated to be 0.24 mM, which is 2-fold higher than that of human GnT-IVa. The K_m values of GnT-IVb for pyridylaminated (PA) acceptor sugar chains with different branch numbers were 3- to 6-fold higher than those of GnT-IVa. To compare substrate specificities more precisely, we generated recombinant soluble enzymes of human GnT-IVa and GnT-IVb with N-terminal flag tags. Both enzymes showed similar substrate specificities as determined using fourteen PA-sugar chains. They preferred complex-type *N*-glycans over hybrid-types. Among the complex-type *N*-glycans tested, the relative activities of both enzymes were increased in proportion to the number of GlcNAc branches on the Man α1-6 arm. The Man α1-6 arm of the acceptors was not essential for their activities because a linear pentasaccharide lacking this arm, GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4 GlcNAc-PA, was a substrate for both enzymes. These results indicate that human GnT-IVb exhibits the same acceptor substrate specificities as human GnT-IVa, although GnT-IVb has lower affinities for donors or acceptors than GnT-IVa. This suggests that GnT-IVa is more active than GnT-IVb under physiological conditions and that it primarily contributes to the biosynthesis of *N*-glycans.

Keywords Human GnT-IVa · Human GnT-IVb · Kinetic properties · Substrate specificities · *N*-glycan biosynthesis

Abbreviations

GnT	*N*-acetylglucosaminyltransferase
PA	2-aminopyridine
Gal	D-galactose
GlcNAc	*N*-acetylglucosamine
Man	D-mannose
Fuc	L-fucose
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

UDP-*N*-acetylglucosamine: α1,3-D-mannoside β1,4-*N*-acetylglucosaminyltransferse (GnT-IV) (EC2.4.1.145) catalyzes the transfer of GlcNAc from UDP-GlcNAc to the GlcNAcβ1-2Manα1-3 arm of the core structure of *N*-glycans through a β1-4 linkage. This enzyme is essential for producing the C-2,4 branch on complex- and hybrid-type *N*-glycans. The products of GnT-IV are normally found in certain glycoproteins, such as erythropoietin [1] and

S. Oguri (✉)
Department of Bioproduction, Faculty of Bioindustry, Tokyo University of Agriculture, 196 Yasaka, Abashiri, Hokkaido 099-2493, Japan
e-mail: s-oguri@bioindustry.nodai.ac.jp

A. Yoshida · M. Takeuchi
Central Laboratories for Key Technology, Kirin Brewery Co., Ltd., 1-13-5 Fukuura, Kanazawaku, Yokohama 236-0004, Japan

M. T. Minowa
Central Laboratories for Key Technology, Kirin Brewery Co., Ltd., 1-13-5 Fukuura, Kanazawaku, Yokohama 236-0004, Japan; Life Science Group, Hitachi, Ltd., 1-3-1 Minamidai, Kawagoe, Saitama, 350-1165, Japan

α1-acid glycoprotein [2]. However, human chorionic gonadotropin (hCG) from choriocarcinoma patients, but not normal individuals, has been found to be modified with GnT-IV products [3,4]. These observations suggest that GnT-IV activity is elevated in choriocarcinoma. Similarly, γ-glutaminyltranspeptidase from human hepatoma tissue has more GlcNAc branches by GnT-IV than from normal tissues [5]. The upregulation of GnT-IV activity has been also reported in human pancreatic carcinoma [6]. We previously purified and cloned GnT-IV from bovine small intestine [7,8]. Using the bovine cDNA, two homologous cDNAs were cloned from human and were named GnT-IVa and GnT-IVb [9,10]. The deduced amino acid sequences of human GnT-IVa and GnT-IVb comprise 535 amino acids and 548 amino acids, respectively, and they share 62% identity. Northern analysis of normal human tissues revealed that GnT-IVb is widely expressed, whereas GnT-IVa is restricted to a few tissues, including thymus, pancreas, spleen, peripheral blood leukocytes, and small intestine [9,10]. Southern analysis proved that most animals possess both GnT-IV genes, and the initially purified bovine enzyme was identified as bovine GnT-IVa [9,10].

The question has been raised as to which isozyme is upregulated during oncogenesis. We previously revealed that GnT-IV activities in three different choriocarcinoma cell lines are increased 16- to 66-fold over those in normal placentas and that the mRNA for GnT-IVa but not GnT-IVb is strongly expressed in all of the cell lines [11]. Moreover, we found that the hCG produced by choriocarcinoma cells exhibiting higher GnT-IV activity carries C-2,4 branched complex-type *N*-glycans [12]. Recently, it was reported that upregulation of the GnT-IVa gene expression is closely associated with metastatic potential in primary colorectal carcinoma [13].

The substrate specificities and enzymatic properties of GT-IVa were characterized using the purified bovine enzyme [7], but the substrate specificity of human GnT-IVa and GnT-IVb has not yet been determined. In the current studies, we compared the kinetic properties of the GnT-IV isozymes to elucidate their roles in *N*-glycan biosynthesis.

Materials and methods

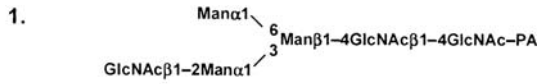
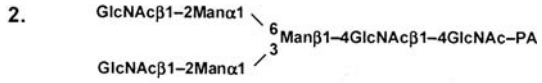
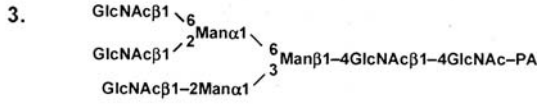
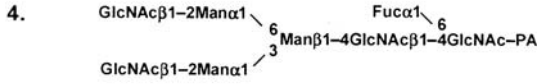
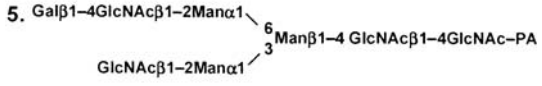
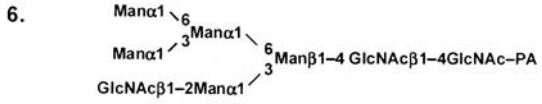
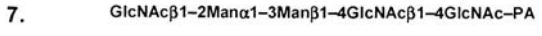
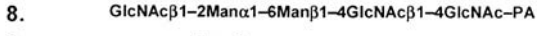
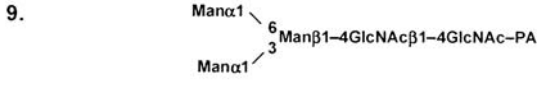
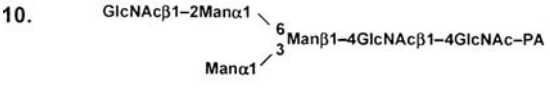
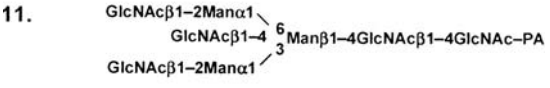
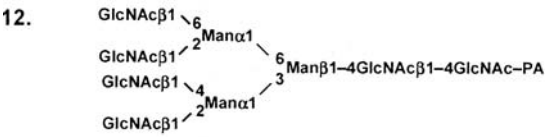
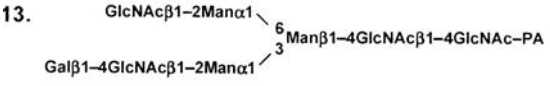
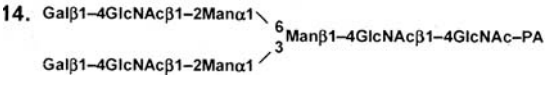
Materials

UDP-GlcNAc was purchased from Sigma (St. Louis, MO). Sialidase from *Arthrobacter ureafaciens* was purchased from Nacalai Tesque (Kyoto, Japan). β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase from jack bean were purchased from Seikagaku Co. (Tokyo, Japan). Recombinant *N*-glycosidase F was purchased from Roche Diagnostics (Mannheim, Germany). Several PA sugar chains were purchased from Takara Shuzo (Kyoto, Japan) and used as standards.

Preparation of acceptor sugar chains

The structures of all sugar chains used in the assay are shown in Table 1. Acceptor 1 was prepared from bovine fibrinogen (Fraction I; Sigma) according to the method of Tokugawa *et al* [14]. Acceptor 6 was kindly provided by

Table 1 Structures of acceptor oligosaccharides used

	Structures
1.	
2.	
3.	
4.	
5.	
6.	
7.	
8.	
9.	
10.	
11.	
12.	
13.	
14.	

Dr. Y. Chiba (AIST, Tsukuba, Japan). Acceptor 7 was prepared from monosialylated biantennary complex-type PA-labeled *N*-glycan with a sialylated side chain in the Manα1-3 arm by sequential exoglycosidase digestion. To obtain the monosialylated sugar chains, disialylated biantennary PA-labeled *N*-glycan (1 μmol) prepared from human apotransferrin was subjected to limited hydrolysis in 10 mM HCl at 60°C for 60 min. The reaction mixture was then applied to a TSK ODS-80 Tm column (4.6 mm × 250 mm, Tosoh, Tokyo, Japan), and sugar chains were eluted at 40°C with a linear gradient of 1-butanol (0.15% to 0.5%) in 100 mM acetate-triethylamine buffer (pH 5.0) at a flow rate of 1.0 ml/min for 35 min. Di-, mono- (in a Manα1–3 branch and a Manα1-6 branch, respectively), and desialylated sugar chains were separated into four peaks. The yield of each sugar chain was 30%, 25%, 27%, and 18%, respectively. The monosialylated biantennary complex-type *N*-glycan with a sialylated side chain on Manα1-3 arm (200 nmol) was sequentially digested with various exoglycosidases in 500 μl of 0.15 M citrate-phosphate buffer (pH 5.0) in the following order: 5U β-galactosidase, 1U sialidase, 5U *N*-acetylhexosaminidase, and 3U β-galactosidase. Each digestion was carried out at 37°C overnight in a toluene atmosphere, and reactions were terminated by boiling for 5 min. After the series of digestions, the resulting sugar chain was desalted by reverse-phase chromatography using a C18 cartridge column (Sep-Pak light, Waters, Milford, MA) and then digested with 10U α-mannosidase in 200 μl 0.1 M sodium acetate buffer (pH 4.5) containing 2 mM $ZnCl_2$ and 0.1 mg/ml bovine serum albumin. The reaction product was purified on a TSK ODS-80 Tm column (4.6 mm × 150 mm, Tosoh). The M_r of the sugar chain was determined by MALDI-TOF-MS analysis. Acceptor sugar chain 8 (Table 1) was prepared using the same method from monosialylated complex-type biantennary PA-labeled *N*-glycan with a sialylated side chain on the Manα1-6 arm. Other sugar chains were prepared as previously described [7].

Determination of GnT-IV activities

The GnT-IV assay was performed as described previously [7]. The standard reaction was carried out in 20 μl containing 20 mM UDP-GlcNAc, 7.5 mM $MnCl_2$, 200 mM GlcNAc, 0.5% Triton X-100, 10% glycerol, 5 mg/ml bovine serum albumin, 125 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.3), 6 μl of enzyme solution, and 0.8 mM acceptor substrate. After incubation at 37°C for 120 min, the products were separated by reverse-phase high performance liquid chromatography (TSK ODS-80 Tm column; 4.6 mm × 150 mm, Tosoh) at 50°C in 50 mM ammonium acetate buffer (pH 4.0) containing 0.15% 1-butanol at a flow rate of 1.2 ml/min. The eluted sugar chains were detected with a fluorescence detector using excitation and emission wavelengths of 320 and 400 nm, respectively. The purchased acceptor 2 (Table 1) was used as the standard.

Kinetic parameters were estimated from [substrate]/ velocity *vs.* [substrate] plots, which were constructed by varying each acceptor substrate concentration while keeping the UDP-GlcNAc concentration at 20 mM. The acceptor concentrations used were as follows: acceptor 1, 0.2 to 12 mM; acceptor 2, 0.2 to 4.0 mM; and acceptor 3, 0.1 to 4.0 mM. The incubation period was 60 min. Similarly, to estimate the K_m for UDP-GlcNAc, the nucleotide sugar concentration was varied between 0.05 and 2 mM, and the concentration of acceptor 2 was maintained at 0.8 mM.

For determination of acceptor substrate specificities, the incubation was performed using the standard incubation conditions but with 0.1 mM of each acceptor. After incubation at 37°C for 60 min, 20 μl of water was added, and the enzyme reaction was stopped by boiling for 2 min. After filtration, 5 μl of the reaction mixture was applied to a TSK ODS-80 Tm column. The sugar chains were eluted at 50°C with a linear gradient of 1-butanol (0% to 0.5%) in 100 mM ammonium acetate (pH 4.0) at a flow rate of 1.0 ml/min for 50 min.

Transient expression of human full-length GnT-IVa and GnT-IVb cDNAs in COS7 cells

The isolation of cDNAs encoding human GnT-IVa and GnT-IVb was described previously [9,10]. The entire open reading frame of each human GnT-IV gene was subcloned into the pSVL expression vector (Amersham Biosciences, Piscataway, NJ). Ten μg of each expression plasmid was incorporated into 5 × 10^6 of COS7 cells in 0.8 ml of phosphate-buffered saline by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA) at 1600 V and 25 μF. After 72 h of cultivation at 37°C in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen), the cells were collected, suspended in 100 ml of buffer (5 mM Tris-HCl, pH 7.5, and 1 mM phenylmethylsulfonyl fluoride), and disrupted by sonication. The supernatants were collected by centrifugation at 2,000 × *g* for 5 min at 4°C and used in assays of GnT-IV activity. The total protein concentrations were measured using the BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. The specific activity of GnT-IV was expressed as moles of the enzymatic products per min of incubation per mg of protein in the cell homogenate.

Preparation of soluble human flag-GnT-IVa and flag-GnT-IVb

Soluble forms of GnT-IVa and GnT-IVb tagged with the 9-amino acid N-terminal FLAG epitope (MDYKDDDDK) were secreted from HEK293T cells by attaching the trypsin secretion signal. These constructs were designated

flag-GnT-IVa and flag-GnT-IVb, respectively. The DNA encoding the secretion signal, FLAG-tag, and soluble portion (Asp88-Asn535) of GnT-IVa was prepared from pFLAG-CMV-1 (Sigma) and human GnT-IVa cDNA [9] and was inserted into pcDNA3.1 Neo(+) (Invitrogen). An expression plasmid for the soluble portion (Asp96-Asp548) of human GnT-IVb [10] was constructed in a similar fashion. The resulting expression plasmid was transfected into HEK293T cells [15]. The culture supernatant was collected after 2 days, and used in assays of GnT-IV activity. The protein contents of FLAG-tagged recombinant enzymes in culture medium were determined by the dot-blotting method according to the manufacture's instructions. Culture media containing the secreted flag-GnT-IVa and flag-GnT-IVb were serially diluted with 0.2 mg/ml bovine serum albumin (BSA) in 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl solution (TBS) and were applied onto a nitrocellulose membrane. After blocking with 5% BSA in TBS containing 0.05% Tween 20 (TBS-T) overnight at 4°C, the membrane was incubated with 0.26 μg/ml mouse anti-flag M1 antibody (Sigma) in TBS-T containing 5 mM $CaCl_2$ for 1 h at room temperature. After washing with TBS-T, the membrane was incubated with 0.5 μg/ml horseradish peroxidase conjugated goat anti-mouse IgG (Sigma) for 1 h at room temperature. The reactive protein dots were visualized by a chemiluminescence using an ECL-plus kit (Amersham Biosciences). The protein dots were scanned and the signal intensity was densitometrically quantified. Amino-terminal Met-FLAG-BAP fusion protein (Sigma) was used for protein standard. The specific activity was expressed as moles of the enzymatic products per min of incubation per mg of the recombinant protein.

N-glycosidase F treatment and immunoblot analysis of flag-GnT-IVa and flag-GnT-IVb

Culture media containing the secreted flag-GnT-IVa and flag-GnT-IVb were concentrated by ultrafiltration (Microcon; Millipore, Billerica, MA) and denatured in 20 μl digestion buffer (50 mM Tris-HCl, pH 7.2, containing 1% mercaptoethanol and 50 mM EDTA) with 1% sodium dodecyl sulfate (SDS) at 100°C for 3 min. After denaturation, the solution was diluted with 50 μl digestion buffer containing 1.25% *n*-octylglucoside. The reaction solution was incubated with 10 U *N*-glycosidase F at 37°C overnight. The reaction was terminated by boiling with the loading buffer containing dithiothreitol. The resulting samples were loaded onto a SDS-polyacrylamide gel electrophoresis (PAGE) gel (7.5% acrylamide), separated under reducing conditions, and then transferred to a polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences). The blot was incubated with a mouse anti-flag M1 antibody. After washing, the blot was reacted with horseradish peroxidase conjugated goat anti-mouse IgG (Sigma) and visualized with a nitro blue tetrazolium substrate kit (Wako Chemical Co., Osaka, Japan).

Purification of flag-GnT-IVa and flag-GnT-IVb

Culture medium supplemented with 5 mM $CaCl_2$ was applied to an anti-FLAG M1 affinity gel column (Sigma) equilibrated with 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 5 mM $CaCl_2$. After washing the column with the same buffer, the soluble flag-GnT-IV was eluted from the column with 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 0.2 mg/ml FLAG peptide (Sigma), and 7.5 mM $MnCl_2$. The purified enzymes were subjected to SDS-PAGE analysis on 7.5% acrylamide gels, and the protein bands were visualized using a silver staining kit (Daiichi Pure Chemicals, Tokyo, Japan).

Results

Kinetic properties of the two GnT-IV isozymes

The entire open reading frames of human GnT-IVa and GnT-IVb in the pSVL vector were transiently expressed in COS7 cells, and the GnT-IV activities in the cell homogenate were determined using bi-antennary PA complex-type sugar chain (acceptor 2, Table 1) as an acceptor substrate. When 10 μg of each GnT-IV cDNA was used for the transfection, the expressed GnT-IV activity of transfectant was determined to be 377 ± 71.6 and 119 ± 35.3 pmol/min/mg for GnT-IVa and GnT-IVb, respectively (mean $\pm$ SD; $n = 4$). These activities were 104- and 33-fold higher than the activity from the mock transfectant (3.61 ± 1.37 pmol/min/mg), respectively.

We next compared the enzymatic properties of human GnT-IV isozymes expressed in COS7 cells. As described previously for bovine GnT-IVa [7], the activities of both human GnT-IV isozymes depended on the concentration of $MnCl_2$, with maximal activities detected in the presence of 5 to 10 mM $MnCl_2$. Both GnT-IV enzymes were slightly active without addition of any metal, but the activities were eliminated by the addition of EDTA (data not shown).

Because our previous study reported that the bovine GnT-IVa enzyme acts on mono-, bi-, and C-2,6-branched triantennary complex-type *N*-glycans [7], we determined the kinetics of both human GnT-IV isozymes using these sugar chains as substrates (Table 2). The K_m values for acceptors of the GnT-IVa enzyme decreased according to the number of GlcNAc branches attached to the core mannosyl residues. This behavior was also observed for GnT-IVb. GnT-IVb showed 3- to 6-fold higher K_m values for acceptors than GnT-IVa, indicating that GnT-IVb has a lower affinity than GnT-IVa toward acceptor sugar chains. The kinetic efficiency ratios (V_{max}/K_m) for both GnT-IV enzymes were the same for the

Table 2 Kinetic properties of recombinant human full-length GnT-IVa and GnT-IVb expressed in COS7 cells

	GnT-IVa			GnT-IVb		
Substrate[a]	K_m (mM)	V_{max} (pmol/min · mg^{-1})	Kinetic efficiency[b] (V_{max}/ K_m)	K_m (mM)	V_{max} (pmol/min · mg^{-1})	Kinetic efficiency[b] (V_{max}/ K_m)
Acceptor 1	3.19	1.17	0.367 (0.4)	10.5	0.792	0.0755 (0.5)
Acceptor 2	0.971	0.934	0.962 (1.0)	5.72	0.887	0.155 (1.0)
Acceptor 3	0.532	1.52	2.86 (3.0)	3.35	1.64	0.490 (3.2)
UDP-GlcNAc	0.118	ND	ND	0.242	ND	ND

[a]The structures of the acceptors are shown in Table 1.
[b]Numbers in parentheses are ratio of kinetic efficiency observed in the acceptor 2.
ND, Not determined.

three acceptors. This result indicates that both human GnT-IV enzymes have the same branch specificity and that the triantennary complex-type *N*-glycan with a C-2,6 branch is the best acceptor for them. The K_m of GnT-IVa for UDP-GlcNAc was determined to be 0.118 mM, which is half of that for the GnT-IVb enzyme.

Acceptor substrate specificities of human soluble flag-GnT-IVa and flag-GnT-IVb

Because both GnT-IV enzymes showed the same branch specificities but different affinities, we carried out a more detailed analysis of their specificities using fourteen PA-labeled sugar chains with various structures. To avoid the consumption of substrates by other GnTs in the cell lysate, we utilized purified, soluble forms of both GnT-IV enzymes. Our previous study reported that the secreted deletion mutant of bovine GnT-IVa lacking the N-terminal 92 amino acid residues maintains its activity when transiently expressed in COS-7 cells [8]. Therefore, we expressed N-terminally FLAG-tagged truncated GnT-IVa (residues 88–535; flag-GnT-IVa) and GnT-IVb (residues 96–548; flag-GnT-IVb).

The production of the recombinant enzymes was confirmed by immunoblotting of the secreted flag-GnT-IVa and flag-GnT-IVb proteins with an anti-FLAG tag antibody (Figure 1A lanes 1 and 3). Furthermore, *N*-glycosidase F treatment caused a shift in the M_r of both proteins (Figure 1A, lanes 2 and 4). The soluble human flag-GnT-IVa and flag-GnT-IVb contain one and two predicted *N*-glycosylation sites, respectively. The observed shifts in M_r for flag-GnT-IVa (M_r 3000) and flag-GnT-IVb (M_r 2700) were similar, suggesting that only one potential *N*-glycosylation site is occupied with *N*-glycan in both recombinant enzymes. Because of their amino acid sequences, M_r values of flag-GnT-IVa and flag-GnT-IVb were predicted to be 52,506 and 53,267, but after *N*-glycosidase F treatment, they had M_r values of 54,900 and 59,000 (Figure 1, lanes 2 and 4). The reason for this discrepancy remains unclear.

Kinetic properties of the secreted both flag-GnT-IV enzymes were examined. Using the standard assay, the specific

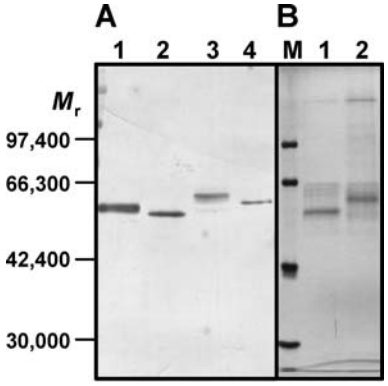


Fig. 1 Expression of recombinant flag-GnT-IVa and flag-GnT-IVb. (A) Aliquots of culture medium containing each secreted recombinant flag-GnT-IV were treated with (lane 2 and 4) or without (lane 1 and 3) *N*-glycosidase F and separated by 7.5% SDS-PAGE. After electrophoresis, the proteins were detected by immunoblotting using an anti-flag antibody. Lanes 1 and 2, flag-GnT-IVa; lanes 3 and 4, flag-GnT-IVb. (B) The purified flag-GnT-IVa (lane 1) and flag-GnT-IVb (lane 2) were analyzed by 7.5% SDS-PAGE. Protein bands were visualized by silver staining. Size standards are shown in lane M.

activities of flag-GnT-IVa and flag-GnT-IVb were determined to be 30.2 and 5.05 nmol/min/mg, respectively. Table 3 shows the kinetic parameters for the recombinant flag-GnT-IV enzymes. The K_m values for the acceptor and donor of both flag-GnT-IV enzymes were slightly higher than those determined for the full-length enzymes.

Both flag-GnT-IV enzymes were purified from the medium using an anti-FLAG antibody column (Figure 1B).

Table 3 Kinetic parameters of recombinant soluble human flag-GnT-IVa and flag-GnT-IVb

		Acceptor 2[a]	
Transferases	UDP-GlcNAc K_m (mM)	K_m (mM)	V_{max} (nmol/min · mg^{-1})
flag-GnT-IVa	0.358	1.04	70.3
flag-GnT-IVb	0.341	6.94	46.6

[a]The structure of acceptor is shown in Table 1.

Table 4 Acceptor specificities of recombinant soluble human flag-GnT-IVa and flag-GnT-IVb
Purified flag-GnT-IVa and flag-GnT-IVb was assayed in the standard mixture except that the concentration of acceptors was 0.1 mM. Relative activity is expressed as percent of activity determined an acceptor 2 as substrate

	Relative activity (%)	
Acceptors[a]	flag-GnT-IVa	flag-GnT-IVb
1	58.6	63.4
2	100	100
3	227	285
4	82.3	81.2
5	44.2	32.1
6	24.9	31.5
7	35.9	41.4

The enzymatic products were not detected in both flag-GnT-IV reactions when the acceptors 8 to 14 were used.
[a]The structures of acceptors are shown in Table 1.

No activity of GnT-I, -II, -III, or -V was detected in the both purified preparations (data not shown). We next investigated the acceptor substrate specificities of the purified recombinant soluble enzymes using a set of PA sugar chains that were derived from *N*-glycans (Table 4). The substrate specificity of flag-GnT-IVa agreed with that reported for purified bovine GnT-IVa [7]. In addition to the reported specificity of bovine GnT-IVa, flag-GnT-IVa could act on the linear pentasaccharide lacking the Manα1-6 residue (acceptor 7). In contrast, the linear pentasaccharide lacking the Manα1-3 residue (acceptor 8) was not an acceptor for human GnT-IVa. Furthermore, the substrate specificity of human GnT-IVb agreed with that of human GnT-IVa. Both GnT-IV enzymes act on hybrid-type *N*-glycan (acceptor 6), but the relative activity against hybrid-type *N*-glycan was lower than those against mono-, bi-, and triantennary complex-type *N*-glycans, indicating that both GnT-IV enzymes prefer complex-type over hybrid-type *N*-glycans.

Discussion

In this report, we used two types of recombinant human GnT-IV enzymes for kinetic analysis: full-length enzymes transiently expressed in COS7 cells and purified, soluble truncated forms tagged with FLAG sequence at their N-terminus. Based on the amino acid sequence, the structures of both GnT-IV enzymes are predicted to be type-II transmembrane proteins [9,10]. N-terminal sequence analysis of purified bovine GnT-IVa further revealed that the purified enzyme preparation contains truncated forms initiating at Ile 93 due to proteolysis during purification. This truncated form lacks the domains between the cytosol and the stem [8]. The N-terminal deletion mutant of bovine GnT-IVa lacking the 92 amino acid residues showed 32% of the activity of the full-length enzyme when the enzymes were transiently expressed in COS7 cells [8]. This observation indicates that the cytoplasmic, transmembrane, and stem domains are not essential for enzyme activity of bovine GnT-IVa; however, the deletion of these domains may affect its kinetic properties. In fact, the estimated K_m values for acceptor and donor of soluble enzymes were higher than those for full-length enzymes transiently expressed in COS7 cells. Based on these observations, we performed enzymatic analyses on full-length enzymes transiently expressed in COS7 cells to accurately determine the branch specificities between human GnT-IVa and GnT-IVb.

The substrate specificities of human flag-GnT-IVa and flag-GnT-IVb were the same, and, as observed in purified bovine GnT-IVa [7], they absolutely required the GlcNAcβ1-2 residues produced by GnT-I on the Manα1-3 arm in the core mannose. Furthermore, the present result clearly showed that the linear pentasaccharide, GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-PA (acceptor 7) was a substrate for GnT-IV enzymes, whereas GlcNAcβ1-2Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA (acceptor 8) was not; in other words, the GlcNAcβ1-2Manα1-3 moiety is essential for their use as an acceptor substrate. In addition, tetraantennary sugar chain (acceptor 12) was not a substrate for either flag-GnT-IV enzymes, which confirms our preliminary finding that human GnT-IVb was not GnT-VI [10], a β-1,4 GnT that acts on the Manα1-6 arm of the core structure [16]. Both human GnT-IV enzymes could act on hybrid-type *N*-glycans. This activity of GnT-IV was first identified in hen oviduct by Allen *et al.* [17], and the product is found in ovalbumin [18]. Our current findings indicate that GnT-IV prefers the complex-type *N*-glycan over the hybrid-type. The relative activity against the monoantennary sugar chain (acceptor 1) of GnT-IV was higher than against the hybrid-type (acceptor 6), suggesting that mannose residues attached on the Manα1-6 arm interfere with GnT-IV action.

Following the discovery of the human GnT-IVb gene, the isolation of genes homologous to GnT-I and GnT-V was reported [19–22]. These gene products show broad acceptor substrate specificities compared to the original enzymes, because they can act on both Manα1-3 and Manα1-6 arms of *N*-glycan [20–22]. Furthermore these homologues catalyze the transfer of GlcNAc to peptide *O*-mannosyl residues. Because GnT-IVb has a strict specificity toward the Manα1-3 arm in *N*-glycans, it is assumed that *O*-mannosyl residues are not substrates for GnT-IVb.

Kinetic analysis showed that both enzymes have higher affinities for acceptor sugar chains with more terminal GlcNAc residues at the nonreduced end. It is assumed that the two enzymes have subsites that specifically bind terminal GlcNAc residues in substrates. The K_m for the best acceptor substrate, triantennary complex-type *N*-glycan (acceptor 3), was 0.532 mM for GnT-IVa and 3.35 mM for

GnT-IVb, respectively. These K_m values were higher than for other branch forming GnTs; the K_m values of GnT-I, -II, -III and -V ranged from 0.021 to 0.25 mM when appropriate substrates are used [23–28]. Because of the higher K_m of both GnT-IV enzymes for acceptors, it is assumed that the concentration of substrate glycoproteins in the Golgi lumen is a limiting factor for branch formation by GnT-IV *in vivo*.

This report presents information about the order of GlcNAc addition by GnTs during the tetraantennary complex-type *N*-glycan formation. The tetraantennary structure is formed on the GnT-I product by the actions of various enzymes, including α-mannosidase II and GnT-II, -IV, and -V [29]. Among them, α-mannosidase II, GnT-II and -V are enzymes which act on Manα1-6 arm of *N*-glycans. Both GnT-IV enzymes could act on the product of GnT-I before these enzymes. However, both GnT-IV enzymes showed their highest kinetic efficiencies against the triantennary sugar chains with the C-2,6 branch produced by GnT-II and -V (Table 2); in other words, the preceding branch formations on Manα1-6 arm by other GnTs promote the actions of both GnT-IV enzymes. On the other hand, the bisecting GlcNAc and the galactosylation of the GlcNAcβ1-2Manα1-3 arm prevented the actions of both GnT-IV enzymes (Table 4). These residues are transferred by GnT-III and β1,4-galactosyltransferase (GalT), respectively. Therefore, to form the C-2,4 branch on complex-type *N*-glycans, both GnT-IV enzymes must act on the GnT-I product before these transferases. The possibility of intracellular competition with GnT-IV and Gal T-I has been described in two independent experimental systems: GalT-I deficient mice [30] and CHO cells that overexpress GalT-I genes [31].

In this report, we found that human GnT-IVb has the same substrate specificity as human GnT-IVa. The question has been raised as to which enzyme contributes to the biosynthesis of multi-antennary complex-type *N*-glycans. The lower K_m values of GnT-IVa for various acceptor sugar chains suggest that GnT-IVa is a more active enzyme than GnT-IVb under physiological conditions. Takamatsu *et al.* compared the mRNA expression levels of GnT-IVa and GnT-IVb among 18 human cancer cell lines derived from several tissues [32]. According to this result, GnT-IV activities correlated with the expression levels of GnT-IVa, whereas GnT-IVb mRNA expression was constitutive among all cell lines regardless of the activity. Moreover, cell lines exhibiting high GnT-IV activity (*e.g.*, HepG2 hepatocellular carcinoma cells and MOLT-4 T cell leukemia cells) carry GnT-IV products on their cell surfaces. These observations along with our present results suggest that GnT-IVa is primarily responsible for the biosynthesis of complex-type *N*-glycans, although GnT-IVb might contribute to the basal GnT-IV activity in cells.

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References

1. Takeuchi, M., Takasaki, S., Miyazaki, H., Kato, T., Hoshi, S., Kochibe, N., Kobata, A.: Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. J. Biol. Chem. **263**, 3657–63 (1988)
2. Yoshima, H., Matsumoto, A., Mizuochi, T., Kawasaki, T., Kobata, A.: Comparative study of the carbohydrate moieties of rat and human plasma alpha 1-acid glycoproteins. J. Biol. Chem. **256**, 8476–84 (1981)
3. Mizuochi, T., Nishimura, R., Derappe, C., Taniguchi, T., Hamamoto, T., Mochizuki, M., Kobata, A.: Structures of the asparagine-linked sugar chains of human chorionic gonadotropin produced in choriocarcinoma. Appearance of triantennary sugar chains and unique biantennary sugar chains. J. Biol. Chem. **258**, 14126–19 (1983)
4. Kobata, A.: Structures, function, and transformational changes of the sugar chains of glycohormones. J. Cell. Biochem. **37**, 79–90 (1988)
5. Yamashita, K., Totani, K., Iwaki, Y., Takamisawa, I., Tateishi, N., Higashi, T., Sakamoto, Y., Kobata, A.: Comparative study of the sugar chains of γ-glutaminyltranspeptidases purified from human hepatocellular carcinoma and from human liver. J. Biochem. (Tokyo) **105**, 728–35 (1989)
6. Nan, B.C., Shao, D.M., Chen, H.L., Huang, Y., Gu, J.X., Zhang, Y.B., Wu, Z.G.: Alteration of *N*-acetylglucosaminyltransferases in pancreatic carcinoma. Glycoconjugate J. **15**, 1033–7 (1998)
7. Oguri, S., Minowa, M.T., Ihara, Y., Taniguchi, N., Ikenaga, H., Takeuchi, M.: 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**, 22721–7 (1997)
8. Minowa, M.T., Oguri, S., Yoshida, A., Hara, T., Iwamatsu, A., Ikenaga, H., Takeuchi, M.: cDNA cloning and expression of bovine UDP-*N*-acetylglucosamine: α1, 3-D-mannoside β1,4-*N*-acetylglucosaminyltransferase IV. J. Biol. Chem. **273**, 11556–62 (1998)
9. Yoshida, A., Minowa, M.T., Takamatsu, S., Hara, T., Oguri, S., Ikenaga, H., Takeuchi, M.: Tissue specific expression and chromosomal mapping of a human UDP-*N*-acetylglucosamine: α1,3-D-mannoside β1, 4-*N*-acetylglucosaminyltransferase. Glycobiology **9**, 303–10 (1999)
10. Yoshida, A., Minowa, M.T., Takamatsu, S., Hara, T., Ikenaga, H., Takeuchi, M.: 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. Glycoconjugate J. **15**, 1115–23 (1998)
11. Takamatsu, S., Oguri, S., Minowa, M.T., Yoshida, A., Nakamura, K., Takeuchi, M., Kobata, A.: Unusually high expression of *N*-acetylglucosaminyltransferase-IVa in human choriocarcinoma cell lines: a possible enzymatic basis of the formation of abnormal biantennary sugar chain. Cancer Res. **59**, 3949–53 (1999)
12. Takamatsu, S., Katsumata, T., Inoue, N., Watanabe, T., Fujibayashi, Y., Takeuchi, M.: Abnormal biantennary sugar chains are expressed in human chorionic gonadotropin produced in the choriocarcinoma cell line, JEG-3. Glycoconjugate J. **20**, 473–81 (2004)
13. D'Arrigo, A., Belluco, C., Ambrosi, A., Digito, M., Esposito, G., Bertola, A., Fabris, M., Nofrate, V., Mammano, E., Leon, A., Nitti, D., Lise, M.: Metastatic transcriptional pattern revealed by gene

expression profiling in primary colorectal carcinoma. Int. J. Cancer **115**, 256–62 (2005)
14. Tokugawa, K., Oguri, S., Takeuchi, M.: Large scale preparation of PA-oligosaccharides from glycoproteins using an improved extraction method. Glycoconjugate J. **13**, 53–6 (1996)
15. Pear, W.S., Nolan, G.P., Scott, M.L., Baltimore, D.: Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. U. S. A. **90**, 8392–6 (1993)
16. Taguchi, T., Ogawa, T., Inoue, S., Inoue, Y., Sakamoto, Y., Korekane, H., Taniguchi, N.: Purification and characterization of UDP-GlcNAc: GlcNAcβ1-6(GlcNAcβ1-2)Manα1-R [GlcNAc to Man]-β1, 4-*N*-acetylglucosaminyltransferase VI from hen oviduct. J. Biol. Chem. **275**, 32598–602 (2000)
17. Allen, S.D., Tsai, D., Schachter, H.: 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**, 6984–90 (1984)
18. Yamashita, K., Tachibana, Y., Kobata, A.: The structures of the galactose-containing sugar chains of ovalbumin. J. Biol. Chem. **253**, 3862–9 (1978)
19. Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., Talim, B., Voit, T., Topaloglu, H., Toda, T., Endo, T.: Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. Dev. Cell. **1**, 717–24 (2001)
20. Zhang, W., Betel, D., Schachter, H.: Cloning and expression of a novel UDP-GlcNAc:α-D-mannoside β1,2-*N*-acetylglucosaminyltransferase homologous to UDP-GlcNAc:α-3-D-mannoside β1,2-N-acetylglucosaminyltransferase I. Biochem. J. **361**, 153–62 (2002)
21. Inamori, K., Endo, T., Ide, Y., Fujii, S., Gu J, Honke, K., Taniguchi, N.: Molecular cloning and characterization of human GnT-IX, a novel β1,6-*N*-acetylglucosaminyltransferase that is specifically expressed in the brain. J. Biol. Chem. **278**, 43102–9 (2003)
22. Kaneko, M., Alvarez-Manilla, G., Kamar, M., Lee, I., Lee, J.K., Troupe, K., Zhang, W., Osawa, M., Pierce, M.: A novel β(1,6)-*N*-acetylglucosaminyltransferase V (GnT-VB). FEBS Lett. **554**, 515–9 (2003)
23. Nishikawa, Y., Pegg, W., Paulsen, H., Schachter, H.: 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**, 8270–81 (1988)
24. Bendiak, B., Schachter, H.: 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**, 5784–90 (1988)
25. Ikeda, Y., Koyota, S., Ihara, H., Yamaguchi, Y., Korekane, H., Tsuda, T., Sasai, K., Taniguchi, N.: Kinetic basis for the donor nucleotide-sugar specificity of β1,4-*N*-acetylglucosaminyltransferase III. J. Biochem. (Tokyo) **128**, 609–19 (2000)
26. Gu, J., Nishikawa, A., Tsuruoka, N., Ohno, M., Yamaguchi, N., Kangawa, K., Taniguchi, N.: Purification and characterization of UDP-*N*-acetylglucosamine: α-6-D-mannoside β1-6*N*-acetylglucosaminyltransferase (*N*-acetylglucosaminyltransferase V) from a human lung cancer cell line. J. Biochem. (Tokyo) **113**, 614–19 (1993)
27. Shoreibah, M.G., Hindsgaul, O., Pierce, M.: Purification and characterization of rat kidney UDP-*N*-acetylglucosamine: α-6-D-mannoside β-1,6-*N*-acetylglucosaminyltransferase. J. Biol. Chem. **267**, 2920–7 (1992)
28. Korczak, B., Le, T., Elowe, S., Datti, A., Dennis, J.W.: Minimal catalytic domain of *N*-acetylglucosaminyltransferase V. Glycobiology **10**, 595–9 (2000)
29. Schachter, H.: The joys of HexNAc. The synthesis and function of *N*-and *O*-glycan branches. Glycoconjugate J. **17**, 465–83 (2000)
30. Kotani, N., Asano, M., Inoue, N., Iwakura, Y., Takasaki, S.: Polylactosamine synthesis and branch formation of *N*-glycans in β1,4-galactosyltransferase-1-deficient mice. Arch. Biochem. Biophys. **426**, 258–65 (2004)
31. Fukuta, K., Abe, R., Yokomatsu, T., Minowa, M.T., Takeuchi, M., Asanagi, M., Makino, T.: The widespread effect of β1,4-galactosyltransferase on *N*-glycan processing. Arch. Biochem. Biophys. **392**, 79–86 (2001)
32. Takamatsu, S., Inoue, N., Katsumata, T., Nakamura, K., Fujibayashi, Y., Takeuchi, M.: The relationship between the branch-forming glycosyltransferases and cell surface sugar chain structures. Biochemistry (Mosc) **44**, 6343–9 (2005)