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β4GalT-II is a key regulator of glycosylation of the proteins involved in neuronal development

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Abstract

Seven members of the human β1,4-galactosyltransferase (β4GalTs) have been identified and characterized by many groups. β4GalTs play important roles in the extension of N- and O-linked glycans involved in several biological events. However, it has not been clear which β4GalTs can act on glycoproteins, such as α-dystroglycan and Notch receptors, involved in neuronal development. To clarify which β4GalTs can function, we determined the enzyme activities toward such motifs and the transcript levels in human normal tissues. Among human β4GalTs, both β4GalT-I and β4GalT-II could act efficiently on all substrates, but the relative activity of β4GalT-II was higher than that of β4GalT-I. Transcript of *β4GalT-I* was widely expressed except for brain, and on the other hand, that of *β4GalT-II* was expressed at high levels in the brain. Thus, these results suggest that among human β4GalTs, β4GalT-II is a major regulator of the synthesis of glycans involved in neuronal development.

Keywords: β4GalT-II; α-Dystroglycan; Notch receptors

The Galβ1-4GlcNAc structure has been found in N-linked and O-linked glycans of several glycoproteins, which play important roles in many biological events. Among those glycoproteins, α-dystroglycan (α-DG) and Notch receptors are biologically important in the development of the nervous system. DG is a highly glycosylated component of the dystrophin–glycoprotein complex in muscle and brain [1,2]. α-DG is extracellular subunit of DG and includes *O*-mannosyl oligosaccharides, which are required for binding to laminin, neurexin, and agrin [3–6]. Recently, several congenital muscular dystrophies (CMDs) exhibiting neuronal migration disorders have been reported as defective in the enzymes required for the synthesis of *O*-mannosyl oligosaccharides [6–8]. Notch receptors are glycoproteins, which are modified in epidermal growth factor-like domains by O-linked oligosaccharides [9,10], and play important roles in a wide range of developmental processes. It has been demonstrated that the glycosylation on Notch receptors is essential for normal Notch signaling [11,12], and modulation of glycosylation by

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O-fucosyltransferase 1 (OFUT1) and Fringe changes Notch–ligand interactions [13].

Previously, seven members of the human β1,4-galactosyltransferase (β4GalTs) have been identified and characterized [14–22] in terms of substrate specificity as the following. β4GalT-I is the first isolated galactosyltransferase and acts on non-reducing terminal GlcNAc as an acceptor [14,15]. In the presence of α-lactalbumin, the enzyme can function as lactose synthase [23]. β4GalT-I has been demonstrated to play important roles in poly-*N*-acetyllactosamine extension [24]. β4GalT-II and β4GalT-III act on GlcNAc residues in several glycoproteins and glycolipids, but only β4GalT-II can function as lactose synthase like β4GalT-I, and β4GalT-III efficiently catalyzes the synthesis of the first *N*-acetyllactosamine unit in lactoseries glycolipids [16]. β4GalT-IV acts on neolactoseries glycolipids [17], poly-*N*-acetyllactosamine in core 2 [25], and GlcNAc 6-*O*-sulfate [26]. β4GalT-V has been suggested to be involved in the *O*-glycosylation of core 2 and core 6 [27], and also to participate in the galactosylation of the GlcNAcβ1-6 branch, which is synthesized by GlcNAcT-V [28]. β4GalT-VI has been shown to have lactosylceramide synthase activity [19,20]. β4GalT-VII is galactosyltransferase-I, which is involved in the first galactosylation of the proteoglycan linkage region [21,22]. Although the substrate specificities of these seven β4GalTs have been extensively studied as above, it remained unclear which enzymes can act on a disaccharide (GlcNAcβ1-2Man) on α-DG and a disaccharide (GlcNAcβ1-3Fuc) on Notch receptors.

In the present study, we examined which human β4GalTs act on each of the above substrates and determined their transcript levels in normal tissues. Then, we discussed the possibility that β4GalT-II is a candidate for a key enzyme in neuronal development.

Materials and methods

Construction of human β4GalTs expression vector and purification of FLAG-tagged recombinant human β4GalTs enzyme. The putative catalytic domain of human β4GalTs enzyme (β4GalT-I, 52–398a.a.; β4GalT-II, 42–372a.a.; β4GalT-III, 31–393a.a.; β4GalT-IV, 55–344a.a.; β4GalT-V, 46–388a.a.; β4GalT-VI, 37–382a.a.; β4GalT-VII, 60–327a.a.) was expressed as a secreted protein fused with a FLAG tag in *Sf21* cells according to the instruction manual of GATEWAY Cloning Technology (Invitrogen). A ~1.3 kb DNA fragment was amplified by two-step PCR. The first PCR used the Bluescript containing each human *β4GalTs* DNA as a template, and the following primers: *T-I*, forward 5′-AAAAAGCAGGCTCCCAACTGGTCG GAGTCT-3′ and reverse 5′-AGAAAGCTGGGTCAAAACGCTA GCTCGGTG-3′; *T-II*, forward 5′-AAAAAGCAGGCTTCTTCAGC CGCTTCAGTG-3′ and reverse 5′-AGAAAGCTGGGTCCGAGA GCCTCTGTCCAT-3′; *T-III*, forward 5′-AAAAAGCAGGCTGCT TCCGAAGTCTCAGTG-3′ and reverse 5′-AGAAAGCTGGGTCC CCATGAATTCGGTTTC-3′; *T-IV*, forward 5′-AAAAAGCAGGC TCCCTCATTTTGGGGAAGG-3′ and reverse 5′-AGAAAGCTG GGTCAGGGTCATGCACCAAAC-3′; *T-V*, forward 5′-AAAAAG CAGGCTTGATGCAAGCCCAAGGCA-3′ and reverse 5′-AGA AAGCTGGGTCGGTGGGTAAAGCAAACG-3′; *T-VI*, forward 5′-AAAAAGCAGGCTCAGGCATCGCCAACACAT-3′ and reverse 5′-AGAAAGCTGGGTTCTACCTTGCCACGACAG-3′; *T-VII*, forward 5′-AAAAAGCAGGCTCTGAGCACTGGGAAGAAG-3′ and reverse 5′-AGAAAGCTGGGTCACTGTCCATCCAGCTCA-3′. The second PCR used the first PCR product as a template, the forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′, and the reverse primer 5′-GGGGACAAGTTTGTACAAAAAAGC AGGCT-3′. The forward and reverse primers were flanked with *att*B1 and *att*B2 sequences, respectively, to create the recombination sites. The amplified fragment was recombined between the *att*P1 and *att*P2 sites of the pDONR 201 vector using the BP CLONASE Enzyme Mix (Invitrogen). Then, the insert was transferred between the *att*R1 and *att*R2 sites of pVL1393-FLAG to yield pVL1393-FLAG vector. PVL1393-FLAG is an expression vector derived from pVL1393 (Pharmingen, San Diego, CA) and contains a fragment encoding the signal peptide of human immunoglobulin κ (MHFQVQIFSFLLISASVIMSRG), the FLAG tag (DYKDDDDK), and a conversion site for the GATEWAY system.

PVL1393-FLAG-*β4GalTs* were cotransfected with BaculoGold viral DNA (Pharmingen, San Diego, CA) into *Sf21* insect cells according to the manufacturer's instructions and incubated for 3 days at 27 °C to produce recombinant viruses. *Sf21* cells were infected with recombinant viruses at a multiplicity of infection of five and incubated for 72 h at 27 °C. The culture supernatants were harvested and mixed with 100 μl anti-FLAG M1 AFFINITY GEL (Sigma). The protein–gel mixture was washed twice with 50 mM Tris-buffered saline (50 mM Tris–HCl, pH 7.4, and 150 mM NaCl) containing 1 mM $CaCl_2$ and eluted with 100 μl of 100 μg/ml FLAG peptide in 10 mM Tris-buffered saline (Sigma). GalT assay was determined as described below.

Western blot analysis. The enzymes purified above were subjected to 12.5% SDS–polyacrylamide gel electrophoresis, followed by Western blot analysis. The separated proteins were transferred to a Hybond-P membrane (Amersham Bioscience). The membrane was probed with anti-FLAG M2-peroxidase conjugate (Sigma) and stained with Konica Immunostaining HRP-1000 (Konica, Tokyo, Japan). The intensity of positive bands on Western blotting was measured by a densitometer to determine the amount of the purified enzyme using FLAG-BAP Control Protein (Sigma).

Assay of GalT activity toward fluorescein labeled substrate. To determine the enzyme activity, UDP-Gal (Sigma) was utilized as a donor substrate and for various acceptor substrates. The synthesis of GlcNAcβ1-3Fuc-dansyl (DNS) is described below. Glycosylation of 2-(trimethylsilyl)ethyl 2,4-di-*O*-benzyl-β-L-fucopyranoside with phenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside using *N*-iodosuccinimide-trifluoromethanesulfonic acid as a promoter gave the disaccharide derivative in 78% yield. The disaccharide derivative was converted into phenyl thioglycoside, via removal of the phthaloyl group, N-acetylation, cleavage of 2-(trimethylsilyl)ethyl group, O-acetylation, and reaction with thiophenol in the presence of $BF_3 \cdot OEt_2$. Glycosylation of 2-(benzyloxycarbonylamino)ethanol with the phenyl thioglycoside of the disaccharide using dimethyl(methylthio)sulfonium triflate as a promoter gave the α-glycoside (72%), which was transformed, via de-O-acetylation, reductive removal of benzyl groups and benzyloxycarbonyl group, and coupling of 6-(dansylamino)hexanoic acid succinimidyl ester into the desired substrate GlcNAcβ1-3Fuc-DNS.

GlcNAcβ1-2Man was obtained from Honen (Tokyo, Japan). GlcNAcβ1-3(Galβ1-4GlcNAcβ1-3)$_2$-2AB(2-aminobenzamide) [29] and GlcNAcβ1-2Man-2AB were prepared by labeling the corresponding oligosaccharides with 2AB according to the instruction manual of the Signal 2AB glycan labeling kit (Oxford GlycoScience, UK).

For the reaction in the GalT assay, 14 mM Hepes (pH 7.4) containing 0.1% Triton X-100, 0.25 mM UDP-Gal, 11 mM $MnCl_2$, and 0.6 mM acceptor substrate was used. A 5 μl volume of enzyme solution

was added to 15 μl of each reaction mixture and incubated at 37 °C for 1 h. After the mixture was filtrated with an Ultrafree-MC column (Millipore, Bedford, MA), a 15 μl aliquot was subjected to phase-high performance liquid chromatography (HPLC) on an ODS-80Ts QA column (4.6 × 250 mm; Tosoh, Tokyo, Japan) and a GlycoSep N amide-adsorption column (GlycoSystems, Oxford) for DNS labeled substrate and 2AB labeled substrate, respectively. Acetonitrile (30%)/H_2O (70%) and 35% ammonium formate (pH 4.4)/65% acetonitrile were used as running solution at a flow rate of 1.0 ml/min at 30 °C for the analyses of DNS labeled substrate and 2AB labeled substrate, respectively. SCL-10A_{vp} (Shimazu, Kyoto, Japan) was used for detection of the peaks.

Assay of GalT activity toward GlcNAcβ1-2mannosylpeptide. The GalT activity was based on the amount of [^{3}H]Gal transferred to a GlcNAcβ1-2mannosylpeptide, Ac-Ala-Ala-Pro-(GlcNAcβ1-2Man) Thr-Pro-Val-Ala-Ala-Pro-NH_2. The acceptor substrate was prepared by the enzymatic reaction, GlcNAc transferred to a mannosylpeptide, Ac-Ala-Ala-Pro-(Man)Thr-Pro-Val-Ala-Ala-Pro-NH_2, using human protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) as described previously [7]. Briefly, a reaction mixture contained 140 mM Mes buffer (pH 7.0), 400 mM UDP-GlcNAc, 400 mM mannosylpeptide, 10 mM $MnCl_2$, 2% Triton X-100, 5 mM AMP, 200 mM GlcNAc, 10% glycerol, and partially purified recombinant human POMGnT1 in 50 μl total volume. After being incubated for 72 h at 37 °C, the GlcNAcβ1-2mannosylpeptide was separated by a Wakopak 5C18-200 column (4.6 × 250 mm; Wako Pure Chemical Ind., Osaka). Solvent A was 0.1% trifluoroacetic acid in distilled water and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The peptide was eluted at a flow rate of 1 ml/min using a linear gradient of 1–25% solvent B and monitored continuously at 215 nm.

The GalT assay was performed in a mixture of 10 mM Hepes buffer (pH 7.4), 0.2 mM UDP-[^{3}H]Gal (240,000 dpm/nmol; New England Nuclear, Boston, MA), 0.4 mM acceptor substrate (GlcNAcβ1-2mannosylpeptide), 10 mM $MnCl_2$, and purified enzyme solution in 50 μl total volume. After being incubated for 2 h at 37 °C, the product was separated by HPLC as described above and the radioactivity of each fraction was measured using a liquid scintillation counter.

Quantitative analysis of human β4GalTs transcript in human normal tissues by real time PCR. Total RNA was extracted from human tissues by the methods of Chomczynski and Sacchi. First-strand cDNA was synthesized using a SuperscriptII first-strand synthesis kit (Invitrogen) according to the manufacturer's instructions. Quantitation of each human *β4GalTs* transcript expression was performed by real time PCR using the following primers and probe: *T-I*, forward 5′-TCACAAGGTGGCCATCATCA-3′, reverse 5′-GCAGGACTGGGTGCAAATAATAT-3′, and probe 5′-TCCATTCCGCAACCGGCAGG-3′; *T-II*, forward 5′-CAACCAGCATGGTGAGGACA-3′, reverse 5′-AGCGCCTCTAGGAAGCCC-3′, and probe 5′-CAACCGGGCCAAGCTGCTTAACG-3′; *T-III*, forward 5′-CCATGTTGCCGTTGCTATGA-3′, reverse 5′-AGTGCTGAGACTCCTCCGAAGT-3′, and probe 5′-TGGATACAGCCTCCCGTACCCCCA-3′; *T-IV*, forward 5′-ACTTGACAACTGCCCTTCTGTGT-3′, reverse 5′-CAAAGTGAGATCTGGTTTGAAAA-3′, and probe 5′-TCCTTACTCAGAGGCCAGAGCAAGCT-3′; *T-V*, forward 5′-CGCTGCTGTACTTCGTCTATGTG-3′, reverse 5′-CCTTGGGCTTGCATCATGA-3′, and probe 5′-CGCCCGGCATAGTGAACACCTACCT-3′; *T-VI*, forward 5′-CAGAAGCAGCGGCTGGAA-3′, reverse 5′-TCGCACGGTTAAAAGGTTGTG-3′, and probe 5′-GCCAGTCTGTTCAATGACATAAAACGCAA-3′; *T-VII*, forward 5′-CCACATCTACGTGCTCAACCA-3′, reverse 5′-TCTCCAGGAAGCCCACGTT-3′, and probe 5′-TGGACCACTTCAGGTTCAACCGGG-3′. The probe was labeled at the 5′-end with the reporter dye 3FAM, and at the 3′-end with the quencher dye TAMRA (Applied Biosystems, Foste City, CA). Real time PCR was performed using a TaqMan Universal PCR Master Mix (Applied Biosystems). The relative amount of each human *β4GalTs* transcript was normalized by *GAPDH* transcript in the same cDNA.

Mutation analysis of β4GalT-I and β4GalT-II in the patients with CMD. We analyzed genomic DNA from 50 patients with CMD, brain malformation, and ocular abnormalities who have no *fukutin* or *POMGnT1* mutation. Primers to amplify each exon and surrounding intronic sequences were designed from the genomic sequence of the *β4GalT-I* and *β4GalT-II* genes. PCR products from patient genomic DNA were sequenced using Bigdye terminators (Applied Biosystems). Fragments were electrophoresed on an ABI Prism 3100 sequencer (Applied Biosystems).

Results and discussion

Acceptor substrate specificity of human β4GalTs

To facilitate the enzymatic analysis of glycosyltransferases, a soluble form of the protein was generated with immunoglobulin κ signal sequence and a FLAG tag, as described under Materials and methods. The soluble glycosyltransferase was expressed in *Sf-21* cells as a recombinant enzyme fused with the FLAG tag. The fused enzyme expressed in the cell culture supernatants was purified by anti-FLAG M1 antibody-conjugated resin and quantitated by Western blotting analysis using anti-FLAG antibody.

We determined the acceptor substrate specificity of the purified human β4GalTs by utilizing three oligosaccharide acceptor substrates, such as a disaccharide (GlcNAcβ1-2Man), a disaccharide (GlcNAcβ1-3Fuc), and poly-*N*-acetyllactosamine, and each product was analyzed on HPLC. The activities of the human β4GalTs toward three acceptor substrates are summarized in Table 1. Among human β4GalTs, β4GalT-II transferred Gal most efficiently to all substrates, and β4GalT-I was the second most efficient. The activity of β4GalT-II was about three times higher than that of β4GalT-I. Then, we examined the GalT activity toward a glycopeptide (GlcNAcβ1-2Man-peptide), to investigate the effect of peptide on specificity of the GalT activity. As a result in Fig. 1, β4GalT-II also transferred Gal most efficiently as mentioned above, although the relative activity of β4GalT-I toward β4GalT-II was higher than the above three substrates, suggesting that the peptide has a role for the specificity of β4GalTs.

β4GalT-II transferred Gal most efficiently toward both *O*-mannosyl glycans and *O*-mannosyl peptide, and β4GalT-I transferred the second most efficiently (Table 1 and Fig. 1). A sialyl *O*-mannosyl glycan, Siaα2-3Galβ1-4GlcNAcβ1-2Man, on α-DG is essential for a laminin-binding ligand, and several CMDs have been reported as being defective in the enzyme required for the synthesis of such *O*-mannosyl glycan [6–8]. Other candidate regulators of *O*-mannosyl glycan, such as Fukutin or Large, have been involved in CMD, but each glycosyltransferase activity has not yet been determined [30,31]. It has been reported that deficiency of *β4GalT-I* causes the congenital disorder of glycosylation (CDG)

Table 1
Acceptor substrate specificity of β4GalTs

Acceptor substrate	nmol/h/mg protein						
	T-I	T-II	T-III	T-IV	T-V	T-VI	T-VII
GlcNAcβ1-2Man-2AB	7.2[a]	25.3	2.5	0.4	0.1	0.6	ND[b]
GlcNAcβ1-3Fuc-DNS	101.0	286.0	8.8	2.0	2.6	17.9	ND
GlcNAcβ1-3(Galβ1-4GlcNAcβ1-3)$_2$-2AB	69.1	173.7	16.9	1.6	0.7	3.2	ND

[a] The values represent averages of two independent experiments.
[b] ND, not detected.

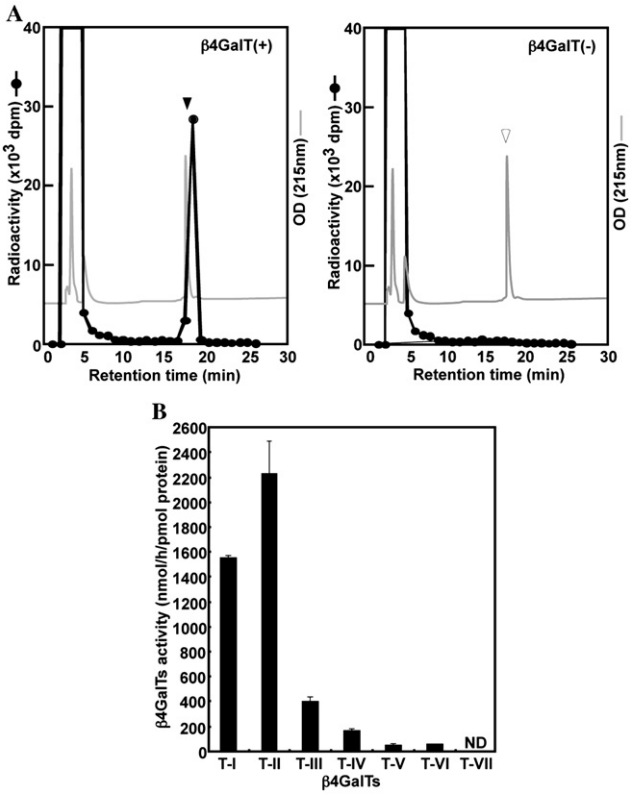

Fig. 1. Activities of human β4GalTs toward GlcNAcβ1-2mannosylpeptide. (A) Reversed phase-HPLC analyses of reaction products from GlcNAcβ1-2mannosylpeptide as an acceptor of UDP-[^{3}H]Gal with (left) or without (right) each human β4GalT. Eluting fractions were collected and their radioactivities were measured with a liquid scintillation counter. Typical example is shown here. The radioactivity and the absorbance of 215 nm are black line and gray line, respectively. The peak at 18.5 min (closed arrowhead and open arrowhead) corresponds to glycopeptide. The GalT activity was determined by incorporation of [^{3}H]Gal into GlcNAcβ1-2mannosylpeptide (closed arrowhead). (B) Activities of human β4GalTs toward GlcNAcβ1-2mannosylpeptide. Data represent the average of two independent experiments and are shown as mean values with standard errors.

type IId, with brain malformation, mental retardation, myopathy, and blood clotting defects [32], but the loss of sialic acid and galactose residues of *O*-mannosyl glycan has not been demonstrated. Also, CMD like phenotypes have not been reported in *β4GalT-I* knockout mice [33,34]. This suggests that β4GalT-II is a possible major regulator of the synthesis of *O*-mannosyl trisaccharide, Galβ1-4GlcNAcβ1-2Man, and may be involved in several CMDs.

Notch receptors are transmembrane glycoproteins, and these *O*-fucosyl glycans are essential for Notch signaling [11,12]. β4GalT acts on a disaccharide (GlcNAcβ1-3Fuc), and the other group demonstrated that among six β4GalTs, only β4GalT-I transferred Gal to the disaccharide in Chinese hamster ovary cells and modulated Notch signaling [35]. However, in our results, β4GalT-I and β4GalT-II transferred Gal efficiently in vitro, and the activity of β4GalT-II was about three times higher than that of β4GalT-I. These differences may be caused by differences of experimental sources such as in vivo or in vitro, and in vivo there is the possibility that the reduction of Notch signaling in *β4GalT-I* mutant cells may be caused by the deficiency of Gal addition to the other Notch signaling related molecules. In *β4GalT-I* knockout mice, growth retardation and semi-lethality before weaning are exhibited [33,34], but these phenotypes are not serious as compared with that of *Lunatic-fringe* deficiency [36], suggesting that another β4GalTs act on the disaccharide. Thus, β4GalT-II is another possible regulator of the elongation of the fringe disaccharide, GlcNAcβ1-3Fuc.

We also examined the GalT activity toward poly-*N*-acetyllactosamine in addition to the two substrates. As shown in Table 1, the activity of β4GalT-II toward poly-*N*-acetyllactosamine was about three times higher than that of β4GalT-I, although β4GalT-I also transferred Gal efficiently. Poly-*N*-acetyllactosamines are often modified to express differentiation antigens and functional oligosaccharides, such as Lewis^X (Le^X), polysialic acid (PSA), and human natural killer-1 (HNK-1) carbohydrate [37,38]. The Le^X Carbohydrate structure functions as a cell–cell recognition molecule in the highly organized structures of the central nervous system [39]. PSA and HNK-1 carbohydrate, both of which are expressed on the outer chain moieties of N-linked oligosaccharides of several neural cell adhesion proteins including the neural cell adhesion molecule [40,41], are involved in neuronal development [42,43]. It was suggested that β4GalT-II and/or β4GalT-V act on PSA and HNK-1 carbohydrate in mouse brain development

[44]. *β4GalT-I* knockout mice demonstrated that PSA and HNK-1 are expressed normally, and no neuronal defects are detected, although reduction of the synthesis of selectin-ligand, such as sialyl Lewis^X, resulted in the reduction of inflammatory responses [45]. Thus, β4GalT-II is a possible major regulator of the synthesis of poly-*N*-acetyllactosamine involved in neuronal development.

As mentioned above, β4GalT-II acted most efficiently on several oligosaccharides involved in the development of the nervous system among human β4GalTs. However, all β4GalTs except for β4GalT-VII show GalT activity toward each substrate, suggesting that several β4GalTs could function coordinately.

Quantitative analysis of human β4GalTs transcript in human normal tissues by real time PCR

We determined the tissue distribution and expression levels of human *β4GalTs* transcript by the real time PCR method, which is a sensitive and accurate assay system. The expression levels of human *β4GalTs* in various tissues were shown as relative values to the *GAPDH* transcript to be able to compare to each other (Fig. 2). Both *β4GalT-II* and *β4GalT-III* were expressed at high levels in brain, and especially in fetal brain (Fig. 2A), suggesting that those genes play important roles in neuronal development. In other reports [16,20], *β4GalT-II* was expressed weakly in adult brain. The discrepancy of *β4GalT-II* expression level in adult brain may be derived from the differences in cDNA sources and the analytical method. The activity of β4GalT-III to the substrates related to neuronal development in this report was much lower than that of β4GalT-II, and β4GalT-III was widely expressed at high levels in all human tissues (Fig. 2B). This suggests that β4GalT-III acts on, not glycans mentioned in this report, but glycolipids that existed in all cells. Moreover, *β4GalT-II* was expressed at highest levels in fetal brain, and this result corresponds with the suggestion of the importance of β4GalT-II in mouse brain development [44].

On the other hand, other *β4GalTs* involving *β4GalT-I* were expressed at very low levels in brain (Fig. 2A) compared with *β4GalT-II* (Fig. 2A), although both *β4GalT-I* and *β4GalT-II* expressed high activity toward substrates in this report as shown in Table 1 and Fig. 1. It was also reported that the levels of *β4GalT-I* did not correlate with the GalT activity levels in mouse brain [46]. These results suggest that β4GalT-II is a major regulator of the synthesis of glycans involved in the brain.

In other normal tissues, *β4GalTs* were expressed differentially at various levels (Fig. 2B). The distribution of each transcript corresponds with other reports [20,22], although there may be individual differences in cDNA sources. High steady state levels of *β4GalT-II* were seen not only in brain, but also in skeletal muscle and testis. The distribution corresponds with that of protein *O-mannosyltransferase* 1 [47], which is involved in the synthesis of *O*-mannosyl glycan, and cause gene of Walker–Warburg syndrome characterized by CMD. So, transcript level and activity of *β4GalT-II* support the possibility that β4GalT-II acts on *O*-mannosyl glycan, and β4GalT-II may be involved in uncharacterized CMDs.

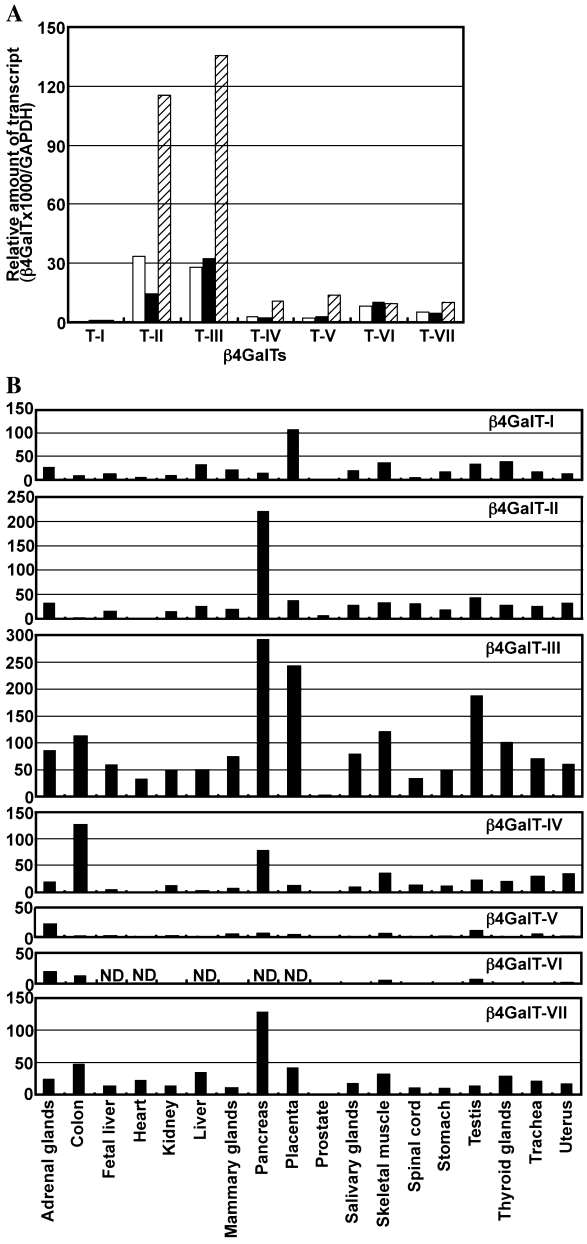

Fig. 2. Quantitative analysis of human *β4GalTs* transcript in human tissues by real time PCR. (A) The expression levels in brain (open bars, whole brain; solid bars, cerebellum; hatched bars, fetal brain). (B) The expression levels in 18 tissues. The expression levels of human *β4GalTs* transcripts were normalized to those of the *GAPDH* transcripts, which were measured in the same cDNAs, and their expression levels could be compared with each other. Two independent experiments were conducted and representative results are shown.

Analysis of β4GalT-I and β4GalT-II in the patients of CMDs

We examined the presence of mutations on the *β4GalT-I* and *β4GalT-II* genes of patients with CMDs whose causing gene was not identified, but we have detected no mutations in any of the 50 patients. As described above, CDG-IId has been observed in only one patient so far [32]. A possible explanation for the absence of *β4GalT-I* and *β4GalT-II* mutations in our subjects is that such patients may not be diagnosed as CMD because of their moderate symptoms. Another possibility is that patients with *β4GalT-I* and *β4GalT-II* mutations were simply not included in the 50 CMD patients studied here. A worldwide survey of the occurrence of *β4GalT-I* and *β4GalT-II* mutations is needed to determine whether any CMD is present to be caused by *β4GalT-I* and *β4GalT-II* mutations.

Conclusions

In the present study, we determined the acceptor specificity of human β4GalTs by utilizing substrates that are essential glycans for neuronal development, and the transcript expression levels in human normal tissues. Among human *β4GalTs*, *β4GalT-II* expressed the highest activity toward all four substrates. Moreover, the transcript levels of *β4GalT-II* were high in brain. These results suggest that β4GalT-II acts most efficiently of all human β4GalTs on glycans involved in neuronal development, such as a disaccharide (GlcNAcβ1-2Man) on α-DG and a disaccharide (GlcNAcβ1-3Fuc) on Notch receptors.

To determine further the function of β4GalT-II in biological events in neuronal development, *β4GalT-II* knockout mice should be generated in future. It may be necessary to knockout two or more *β4GalTs* in mice to elucidate the unknown function of the glycans involved in biological events as β4GalTs may function coordinately in the same tissues.

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