Synthesis of Poly-N-acetyllactosamine in Core 2 Branched O-Glycans

THE REQUIREMENT OF NOVEL β-1,4-GALACTOSYLTRANSFERASE IV AND β-1,3-N-ACETYLGLUCOSAMINYLTRANSFERASE*

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Poly-N-acetyllactosamine is a unique carbohydrate composed of N-acetyllactosamine repeats and provides the backbone structure for additional modifications such as sialyl Lea. Poly-N-acetyllactosamines in mucin-type O-glycans can be formed in core 2 branched oligosaccharides, which are synthesized by core 2 β-1,6-N-acetylgalactosaminyltransferase.

Using a β-1,4-galactosyltransferase (β4Gal-TI) present in milk and the recently cloned β-1,3-N-acetylgalactosaminyltransferase, the formation of poly-N-acetyllactosamine was found to be extremely inefficient starting from a core 2 branched oligosaccharide, GlcNAcβ1→6-(Galβ1→3)GalNAc→R. Since the majority of synthesized oligosaccharides contained N-acetylgalactosamine at the nonreducing ends, galactosylation was judged to be efficient, prompting us to test novel members of the βGal-T gene family for this synthesis. Using various synthetic acceptors and recombinant βGal-Ts, β4Gal-TIV was found to be most efficient in the addition of α2,6-sialylgalactosereductoGlcNAcβ1→6(Galβ1→3)GalNAc→R. Moreover, β4Gal-TIV, together with β-1,3-N-acetylgalactosaminyltransferase, was capable of synthesizing poly-N-acetyllactosamine in core 2 branched oligosaccharides. On the other hand, β4Gal-TI was found to be most efficient for poly-N-acetyllactosamine synthesis in N-glycans. In contrast to β4Gal-TI, the efficiency of β4Gal-TIV decreased dramatically as the acceptors contained more N-acetyllactosamine repeats, consistent with the fact that core 2 branched O-glycans contain fewer and shorter poly-N-acetyllactosamines than N-glycans in many cells. These results, as a whole, indicate that β4Gal-TIV is responsible for poly-N-acetyllactosamine synthesis in core 2 branched O-glycans.

Mucin-type O-glycans are present in a wide variety of cells and play various roles in different cells. Mucin-type glycoproteins are also present in the plasma membrane, and they are often involved in cell-cell interaction. For example, O-glycans present in eggs were shown to be a receptor for both mouse and sea urchin (2, 3). In granulocytes, monocytes, and certain T lymphocytes, mucin-type O-glycans can carry sia1yl Lea, NeuNAcα2–3Galβ1→4(Fucα1→3)GlcNAc→R, at their termini (4–6). Sialyl Lea and its sulfated form are ligands for E-, P-, and L-selectin (7–11). Importantly, these selectins, in particular P- and L-selectin, preferentially bind to sialyl Lea in a limited number of mucin-type glycoproteins such as PSGL-1 (for P-selectin) and GlyCAM-1 and CD34 (for L-selectin) (12–14). As shown previously, sialyl Lea and its derivatives of O-glycans in blood cells can be only formed on core 2 branches, Galβ1→4GlcNAcβ1→6Galβ1→3GlcNAc→R (4, 5). Recent studies demonstrate that sialyl Lea and sialyl Lea in core 2 branches are highly correlated to tumor invasion and vessel invasion of colon carcinomas (15), probably because tumor cells utilize selectin-carbohydrate interaction for their adhesion.

In patients with immunodeficiency such as Wiskott-Aldrich syndrome, AIDS, and leukemia, leukocytes in the peripheral blood express a substantial amount of core 2 branched oligosaccharides, while leukocytes of normal individuals do not express them (16–19). Most recent studies employing transgenic mice demonstrated that such an overexpression of core 2 oligosaccharides weakens the interactions between T lymphocytes and antigen-presenting cells or B lymphocytes, resulting in reduced immune responses such as delayed type hypersensitivity and immunoglobulin isotype switching (20, 21). It has also been shown that AIDS patients produce antibodies against leukosialin expressing core 2 branched oligosaccharides, possibly causing T lymphocyte depletion in those patients (22, 23). Moreover, the overexpression of a mucin-type glycoprotein carrying those oligosaccharides was shown to interfere with cell adhesion (24), while knockout of the leukosialin gene in mice resulted in hyperimmune responses (25). It has been also shown that poly-N-acetyllactosamine can be extended from core 2 branches, forming poly-N-acetyllactosaminyl-O-glycans (4–6, 26). Poly-N-acetyllactosamine is a unique carbohydrate composed of N-acetyllactosamine repeats (Galβ1→4GlcNAcβ1→3),. Poly-N-acetyllactosamines are susceptible to endo-β-galactosidase and larger than typical N-glycans or O-glycans containing only one N-acetyllactosamine in a side chain. Poly-N-acetyllactosamines provide the backbone structure for additional modifications, which are often cell type-specific oligosaccharides, such as sia1yl Lea (1). These results, as a whole, indicate that core 2 branched oligosaccharides play critical roles in cell-cell interaction.

These results indicate that it is crucial to understand the synthesis of core 2 branched oligosaccharide and its further extension to poly-N-acetyllactosamines. To this end, we have cloned the cDNAs encoding core 2 β-1,6-N-acetylgalactosaminyl-
transferase (C2GnT)
1 that forms a core 2 branch (27) and β-1,3-N-acetylgalactosaminyltransferase (iGnT) that forms poly-
N-acetyllactosamine together with β-1,4-galactosyltransferase (βGal-T) (28).

When we tried to synthesize poly-N-acetyllactosamine on core 2 branched oligosaccharides, iGnT and milk βGal-T (βGal-T) failed to form poly-N-acetyllactosamines. Since the majority of the products contained N-acetylgalactosamine at the nonreducing ends, inefficient galactosylation by βGal-T was a likely cause for the lack of poly-N-acetyllactosamine synthesis in core 2 branched oligosaccharides. These unexpected re-
results prompted us to test if any of the new members of βGal-
Ts, which have been identified recently (29–33), is responsible for poly-N-acetyllactosaminyl extension in core 2 branched oligosaccharides. In this report, we summarize these findings and demonstrate that βGal-TIV (33) is the enzyme involved in poly-N-acetyllactosamine extension in core 2 branched oligo-
saccharides. Moreover, we show that βGal-TIV is a rate-limiting factor and responsible for short poly-N-acetyllactosamine extension in core 2 branched O-glycans.

EXPRESSONAL PROCEDURES

Isolation of cDNA Encoding iGnT and iGnTc—cDNA encoding the iGnT was isolated, as described previously (28). The cDNA inserted in the cloned pAMo vector was digested with HindIII and XhoI and cloned into the HindIII and EcoRV sites of pcDNA3.1 (Invitrogen), resulting in pcDNA-1.0-iGnT as described previously (28). pcDNA-I, harboring cDNA encoding a signal peptide sequence, and the IgG binding domain of Staphylococcus aureus protein A, was constructed as described before (28). The catalytic domain of iGnT was cloned into this vector, resulting in pcDNA-AiGnTc (28).

Expression of the Protein A-iGnT Fusion Vector—pcDNA-AiGnTc and pcDNA-I were separately transfected with Lipotectamine (Life Technologies, Inc.) into COS-1 cells as described previously (28), and 48 h after the transfection the medium was replaced with serum-free medium, Opti-MEM (Invitrogen), and cultured for an additional 24 h. The chimeric iGnT secreted into the Opti-MEM was adsorbed into IgG-Sepharose (Amersham Pharmacia Biotech), and the enzyme bound to the beads was used as an enzyme source (34). Alternatively, the culture medium was concentrated 100-fold (for iGnT alone) or 1000-fold (for poly-N-acetyllactosamine synthesis) by a Centricon 10 (Amicon) and directly used as an enzyme source. In most of the studies, the concentrated culture medium was used, since IgG-Sepharose beads had a low capacity as seen for other glycosyl-
transferases (35, 36). For poly-N-acetyllactosamine synthesis, the ac-

tivity of iGnT in the incubation mixtures was 380 nmol/Ihml using 0.5 mg MalβII→4Glcβ→pNP as an acceptor substrate. As described previously (28), the supernatant from mock-transfected COS-1 cells con-
tained less than one-fifth of the activity compared with that derived from pcDNA-AiGnTc-transfected COS-1 cells.

Isolation of cDNA Encoding βGal-T II, III, IV, and V—Isolation of the cDNAs encoding βGal-TII, IIII, IIII, and V has been described previously (29, 33). Based on the nucleotide se-
quences of these cDNAs, cDNAs encoding catalytic domains of βGal-
TII and IIII have been prepared using RT-PCR as described before (29). The catalytic domain of βGal-TIV was prepared by PCR using the expressed sequence tag sequence (expressed sequence tag 489768) as a template as described previously (33). These cDNAs were cloned into pAcGP67 (Pharmingen) and expressed in insect cells as described be-
fore (33). The supernatants from these transfected insect cells were used as an enzyme source.

βGal-TIV was cloned by PCR based on the published nucleotide se-
quence (32, 33). The cDNA encoding a soluble form of βGal-TIV was prepared by PCR using the obtained cDNA as a template. 5′ and 3′-primers for this PCR were 5′-CCCGATGCTCAAGCTTCTGAGT-
CCGGACG-3′ (BamHI site is underlined) and 5′-CCCTCCGATCTGAC-
TGCCTGGTACTCCTGGACG-3′ (XhoI site is underlined). The result-
ant cDNA encoding codon 49 to the stop codon was cloned into BamHI

1 The abbreviations used are: C2GnT, core 2 β-1,6-N-acetylgalactosami-
nyltransferase; βGal-T, β-1,4-galactosyltransferase; iGnT, a catalytic domain of iGnT.

Oligosaccharides—GlcNAcβ1→6Galβ1→3GalNAcα→p-nitrophen-
ol was purchased from Toronto Research Chemicals. This oligo-
saccharide was enzymatically converted to Galβ1→4GlcNAcβ1→6-
Galβ1→3GalNAcα→pNP as described previously (37). Briefly, GlcNAcβ1→6Galβ1→3GalNAcα→pNP (1.14 mmol) was incubated with 494 milliunits of bovine milk βGal-T (Sigma), 5.8 units of calf intestinal alkaline phosphatase (Boehringer Mannheim), 3.5 mg of UDP-galactose in 300 μl of 50 mM HEPEs buffer, pH 7.5, containing 14 mM MnCl2. After incubation for 16 h at 37 °C, the oligosaccharide synthesized was purified by a C18 reverse-phase Sep-Pak cartridge (Waters). The purity of the oligosaccharide was ascertained by thin layer chromatography using silica gel and the solvent system of dic-
chloromethane/methanol/water (12:7:1, v/v/v). The oligosaccharides were detected by charring after spraying with 10% H2SO4 in ethanol. The amount of βGal-T necessary in this reaction was approximately 30 times more than that for galactosylation of GlcNAcβ1→6Manβ1→3Galβ1→4Glcβ→pNP (28).

The acceptors (Galβ1→4GlcNAcβ1→3Galβ1→4GalNAcβ1→6Manβ1→3Galβ1→4Glcβ→pNP) were treated with E. coli β-galacto-
sidosidase (10 units) in 50 mM Tris buffer, pH 7.4, at 37 °C overnight, resulting in the GlcNAc terminated compounds 9, 10, and 11, respectively. Iso-
lution, purification, and characterization of these products was performed as described above. Detailed procedures of the synthesis will be published elsewhere.2

Partial 1H NMR spectroscopy and matrix-assisted laser desorption ionization-time of flight mass spectrometry. Compounds 6, 7, and 8 contain one, two, and three N-acetyllactosamines, corresponding to those where n = 0, 1, and 2, respectively, in the above structure.

For compounds 6, 7, and 8 the products were treated with E. coli β-
galactosidase (10 units) in 50 mM Tris buffer, pH 7.4, at 37 °C overnight, resulting in the GlcNAc terminated compounds 9, 10, and 11, respectively. Isolation, purification, and characterization of these products was performed as described above. Detailed procedures of the synthesis

2 J. C. McAuliffe, M. Fukuda, and O. Hindsgaul, manuscript in preparation.

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Aliquots was determined by a scintillation counter (39).

**Substrate Specificity of β4Gal-TI, -TII, -TIII, -TIV, and -TV—** Assays of β4Gal-TIV were performed in a 50-μl reaction mixture containing 25 mM Tris-HCl, pH 7.5, 4 mM MnCl₂, 0.1% Triton X-100, 5 mM UDP-[³H]GlcNAc (2 × 10⁶ cpm/nmol) (NEN Life Science Products), and an appropriate acceptor (33). For β4Gal-TI, -TII, and -TIII, 25 mM Tris-HCl, pH 7.5, containing 10 mM MnCl₂ and 0.25% Triton X-100 (29) was used, while 25 mM Tris-HCl, pH 7.0, containing 20 mM MnCl₂ and 10 mM galactono-1,5-lactone was used for β4Gal-TV (31). As an enzyme source for β4Gal-TI, human milk β4Gal-T preparation (Sigma) was directly used.

As acceptors, the following oligosaccharides were used: GlcNAc-β1→6Galβ1→3GalNAcα→pNP for galactosylation of core 2 oligosaccharide and GlcNAcβ1→6Manα1→6Manβ→octyl, GlcNAcβ1→4GlcNAcβ1→6Manα1→6Manβ→octyl, and GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→6Manα1→6Manβ→octyl for galactosylation of an antennary extended from GalNAcβ1→6Manα1→6Manβ branch, which is formed by N-acetylglucosaminyltransferase V. After incubation for 1 h at 37 °C, the reaction mixture was applied to a Sep-Pak column, and the amount of the product was determined by measuring the radioactivity of the eluted product as described above. To compare the enzymatic activity of different β4Gal-T samples, each enzyme preparation was first calibrated using 0.5 mM GlcNAcα→p-nitrophenol (Sigma) as an acceptor. This assay showed that the samples of β4Gal-TI, -TII, -TIII, -TIV, and -TV had the activity of 1.9, 1.36, 1.90, 1.27, and 0.962 (all expressed in n mol/h/m) respectively. The final concentration of each enzyme was adjusted to 30.8 n mol/h/ml in all experiments.

**Poly-N-acetylgalactosamine Formation in Core 2 Oligosaccharide and N-Glycan Oligosaccharide—** To assay poly-N-acetylgalactosamine formation, 0.5 mM Galβ1→4GlcNAcβ1→6Galβ1→3GalNAcα→pNP or Galβ1→4GlcNAcβ1→6Manα1→6Manβ→octyl was incubated with different concentrations of β4Gal-Ts (760 nmol/h/m), [³H]GlcNAc (380 nmol/h/ml), 5 mM UDP-[³H]GlcNAc, and 5 mM UDP-[³H]Gal as described above. There is a slight difference in the optimum incubation conditions between iGnT and various β4Gal-Ts. The optimal conditions for β4Gal-Ts were used, since β4Gal-T was found to be a rate-limiting enzyme.

After incubation for 1 h at 37 °C, the product was purified by a Sep-Pak column as described above. The sample was then lyophilized and subjected to HPLC using a column (4 × 300 mm) of NH₂-bonded silica (Varian Micropak AX-5) using Gilson 306. The column was eluted for 60 min with a linear gradient from a mixture of solvent A (80%) and solvent B (20%) to 100% of solvent B; solvent A is composed of 90% acetonitrile and 10% H₂O, while solvent B is composed of 40% acetonitrile and 60% 15 mM KH₂PO₄ in H₂O (40).

Galactosylation of [³H]GlcNAcβ1→4Galβ1→3GalNAc was synthesized by incubating 5 mM Galβ1→4Galβ1→3GalNAc with β4Gal-TI and β4Gal-TIV—[³H]GlcNAcβ1→4Galβ1→3GalNAc was synthesized by incubating 5 mM Galβ1→4Galβ1→3GalNAc with β4Gal-TI and β4Gal-TIV at 37 °C for 3 h. The product was then isolated and applied to the sample to Bio-Gel P-4 gel filtration as described (28). The synthesized [³H]GlcNAcβ1→4Galβ1→3GalNAc (0.5 mM) was incubated with β4Gal-TI or β4Gal-TIV and nonradioactive UDP-Gal under the same incubation mixture as described above, except that the final concentration of β4Gal-TI or β4Gal-TIV was 80.0 nmol/h/ml as assayed using 0.5 mM GlcNAc as an acceptor. After the incubation, UDP-Gal was removed by QAE-Sephadex gel (41), and the products were analyzed by HPLC using the same conditions described above.

**Analysis of Products by Endo-β-Galactosidase Digestion—** Radioactively labeled products were digested with Escherichia freundii endo-β-galactosidase for 18 h at 37 °C (42). The digests were applied to a column (1.0 × 120 cm) of Bio-Gel P-2 (≤400 mesh) equilibrated with 0.1 mM NH₄HCO₃.

**RESULTS**

**Poly-N-acetylgalactosaminibone Formation in Core 2 Banchored O-Glycan and N-Glycan Acceptor—** Recently, we have cloned a cDNA encoding iGnT that forms poly-N-acetylgalactosamine together with β4Gal-T in lacto-N-neotetraose (28). To determine if the cloned iGnT and milk β4Gal-T, β4Gal-TI, can form poly-N-acetylgalactosaminibones on N- and O-glycans, Galβ1→4GlcNAcβ1→6Manα1→6Manβ→octyl (octyltetrasaccharide) and galactosylated core 2 oligosaccharide were used as acceptors. As shown in Fig. 1A, one to four N-acetylgalactosamine repeats were added to the octyltetrasaccharide. After endo-β-galactosidase digestion, peak 1 produced Galβ1→4GlcNAcβ1→3Gal, while peaks 2–4 produced Galβ1→4GlcNAcβ1→3Gal and GlcNAcβ1→3Gal in a ratio expected from the side chains containing 2–4 N-acetylgalactosamine repeats (Fig. 1, B and C). Notably, all of the products contained galactose at nonreducing termini, as seen in many cells (39, 43–46). From galactosylated core 2 branched oligosaccharide, in contrast, GlcNAcβ1→3Galβ1→4GlcNAcβ1→6GalNAcα→pNP (peak 1) and Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→6Galβ1→3GalNAcα→pNP (peak 2) were produced (Fig. 1D). Peaks 1 and 2 yielded GlcNAcβ1→3Gal and Galβ1→4GlcNAcβ1→3Gal after endo-β-galactosidase treatment, respectively, as expected from their structures (Fig. 1, E and F). In nature, core 2 branched oligosaccharides rarely contain N-acetylgalactosamine at nonreducing termini and are almost exclusively terminated with galactose or sialylated galactose (4–6). Moreover, the addition of N-acetylgalactosamine repeats in the core 2 oligosaccharide was only 14.3% of that in the octyltetrasaccharide, indicating that N-acetylgalactosamine formation was inefficient in the core 2 oligosaccharide. To determine whether β4Gal-T or iGnT is responsible for this unexpected, inefficient synthesis of poly-N-acetylgalactosamine on core 2 branches, iGnT or β4Gal-TI was incubated with appropriate acceptors. The results demonstrated that β4Gal-T can add N-acetylgalactosamine with almost equal efficiency to N-glycan acceptor and core 2 branches (Fig. 2A, B, and C). In contrast, β4Gal-TI exhibited substrate inhibition when the core 2 branched oligosaccharide was used as an acceptor (Fig. 2D) but not toward an N-glycan acceptor (Fig. 2C) or N-acetylgalactosamine β→pNP (Fig. 2E). It should be noted, however, that substrate inhibition for β4Gal-TI was reported when higher than 5 mM benzyl-β-GlcNAc or chitobiose or chitotriose was used (29, 47). When the CHO cell lysate was
used as an enzyme source, the core 2 branched oligosaccharide was galactosylated even at its high concentrations (Fig. 2F), consistent with the fact that core 2 branched O-glycans in CHO cells transfected with C2GnT are fully galactosylated (48). These results, shown in Figs. 1 and 2, combined together, indicate that another β4Gal-T, which is apparently present in CHO cells, is responsible for galactosylation of core 2 branches.

**β4Gal-TIV Is Responsible for Galactosylation of Core 2 Branches**—Recently, it has been demonstrated from several laboratories that there exist at least five β4Gal-Ts in addition to β4Gal-TI (29–33, 49). The above results suggest that one of these newly identified β4Gal-Ts might be involved in galactosylation of core 2 branches. To determine if any of these new members of the β4Gal-T family can form galactosylated core 2 branch, GlcNAcβ1→6Galβ1→3GalNAc→pNP was incubated with each of these new members of the β4Gal-T family. As shown in Fig. 3A, β4Gal-TII, -TIII, and -TV exhibited substrate inhibition toward this substrate as did β4Gal-TI. Similar results were obtained when the concentration of these enzymes was increased 5-fold or decreased 5-fold. This substrate inhibition is most likely the reason why the amount of the final product was very low in β4Gal-TI-directed reaction (see also Fig. 1D). This substrate inhibition occurs probably because β-galactose in the acceptor competes with UDP-Gal.

In contrast, the core 2 branch was efficiently galactosylated by β4Gal-TIV with a $K_m$ of 0.29 mM (Fig. 3B; also see Table I). The identical $K_m$ was obtained when the concentration of β4Gal-TIV was decreased 5-fold as expected. These results indicate that β4Gal-TIV is most likely responsible for galactosylation of core 2 branched oligosaccharides. β4Gal-TIV was not included in the studies, since this enzyme is the least related to β4Gal-TI and shown to synthesize Galβ1→4Glcβ→ceramide from Glcβ→ceramide (49).

We then tested if β4Gal-TIV works efficiently on an N-glycan acceptor, GlcNAcβ1→6Manα1→6Manβ→octyl. The results shown in Fig. 4B showed that β4Gal-TIV added galactose much less efficiently to the N-glycan acceptor than the core 2 branch acceptor. Among other β4Gal-Ts, β4Gal-TI most efficiently galactosylated the N-glycan acceptor (Fig. 4A), indicating that β4Gal-TI is most likely involved in poly-N-acetyllactosamine synthesis in N-glycans.

To address the question of whether the nature of the aglycon attached to the core 2 branched acceptor influences the galactosylation, [3H]GlcNAcβ1→6Galβ1→3GalNAc was enzymatically synthesized using recombinant soluble C2GnT (27),
A Novel β4Gal-TIV in Synthesis of Core 2 O-Glycans

**Table I**

| Substrate                              | β4Gal-TIV | β4Gal-TIV |
|----------------------------------------|-----------|-----------|
|                                       | $K_m$     | $V_{max}$ | $K_m$     | $V_{max}$ |
| GlcNAcβ1-3Galβ1-3GalNAcα-pNP          | 6 (Galβ1-3) GalNAcα-pNP          |
| GlcNAcβ1-6Manα1-6Manβ-ocetyl          | 0.88      | 100%      | 0.29      | 70%       |
| GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-6Manα1-6Manβ-ocetyl | 1.14 | 109% | 4.76 | 35% |
| GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-6Manα1-6Manβ-ocetyl | 1.98 | 103% | 4.98 | 5% |

a These parameters could not be obtained due to substrate inhibition.

b $V_{max}$ for β4Gal-TI and β4Gal-TIV is compared with the $V_{max}$ (98.6 pmol/min) obtained for β4Gal-TI using GlcNAcβ1-6Manα1-6Manβ-ocetyl as an acceptor.

Fig. 4. β4Gal-T activity on core 2 or N-glycan acceptor. A, GlcNAcβ1-6Manα1-6Manβ-ocetyl with varying concentrations was incubated with β4Gal-TI (closed circle), TII (open triangle), TII (open square), and TV (closed square). B, various concentrations of GlcNAcβ1-6Galβ1-3GalNAcα-pNP (solid line) and GlcNAcβ1-6Manα1-6Manβ-ocetyl (dotted line) were incubated with β4Gal-TIV, 5 mM UDP-[3H]Gal was used in all experiments. C and D, [3H]GlcNAcβ1-6Galβ1-3GalNAc was incubated with β4Gal-TI (C) and β4Gal-TIV (D), and products were analyzed by HPLC as described in Fig. 1. Peaks at fraction 33 and 38 correspond to [3H]GlcNAcβ1-6Galβ1-3GalNAc and Galβ1-4[3H]GlcNAcβ1-6Galβ1-3GalNAc, respectively.

UDP-[3H]GlcNAc, and Galβ1-3GalNAc. β4Gal-TI or β4Gal-TIV was then incubated with this synthesized [3H]GlcNAcβ1-6Galβ1-3GalNAc. The results, shown in Fig. 4, C and D, clearly demonstrated that the reaction by β4Gal-TI was still incomplete, since the majority of the acceptor was not galactosylated (see the peak at fraction 33). In contrast, β4Gal-TIV completely galactosylated the acceptor. These results indicate that β4Gal-TIV is the enzyme responsible for galactosylation of core 2 branches and that such a synthesis is not influenced by the nature of aglycons.

Poly-N-acetyllactosamine Synthesis by iGnT and β4Gal-TIV—We then tested if iGnT and β4Gal-TIV together can form poly-N-acetyllactosamine on core 2 branched oligosaccharide or N-glycan oligosaccharide acceptor. As shown in Fig. 5B, the products obtained from the core 2 branched acceptor consisted of (Galβ1-4GlcNAcβ1-3)1 or 2Galβ1-4GlcNAcβ1-6-(Galβ1-3)3GalNAcα-pNP, of which structures were elucidated by endo-β-galactosidase digestion (data not shown). The results are also consistent with our previous finding that iGnT cannot add N-acetylglucosamine to a Galβ1-3GalNAc side chain (28). In contrast, only Galβ1-4GlcNAcβ1-3Galβ1-4-GlcNAcβ1-6Manα1-6Manβ-ocetyl was produced from Galβ1-4GlcNAcβ1-6Manα1-6Manβ-ocetyl (Fig. 5A). These results indicate that β4Gal-TIV, together with iGnT, efficiently formed N-acetyllactosamine repeats on core 2 branched oligosaccharides but not on N-glycans.

It is noteworthy that the size of poly-N-acetyllactosamine in core 2 branched oligosaccharides shown in Fig. 5B was much smaller than that in N-glycan acceptor shown in Fig. 1A, when the same amount of the enzymes was used in both experiments. While four and possibly five N-acetyllactosamine units were added to Galβ1-4GlcNAcβ1-6Manα1-6Manβ-ocetyl (Fig. 1A), only two N-acetyllactosamine units were added as a maximum to Galβ1-4GlcNAcβ1-6Manα1-6Manβ-ocetyl-pNP (Fig. 5B).

These results are consistent with the facts that poly-N-acetyllactosamines on core 2 branched oligosaccharides are shorter than those in N-glycans and that the majority of poly-N-acetyllactosaminyl core 2 O-glycans contains only two N-acetyllactosamine repeats in many cells (4–6, 48).
suggested from the knockout of the N-acetyllactosamines. To determine whether this is the case, one or two N-acetyllactosamine units (4–6, 42–46). Moreover, the amount of poly-N-acetyllactosaminyl N-glycans contain three or more N-acetyllactosamine units (4–6, 42–46). Moreover, the amount of poly-N-acetyllactosaminylated O-glycans is much less than that in N-glycans when N-glycans and O-glycans were analyzed in the same lamp molecules (55) or the same CHO cells (46, 48, 55). The present study also demonstrated that poly-N-acetyllactosaminylated O-glycans are resistant toward a glycolipid or glycoprotein acceptor without an aglycon and found that β4Gal-TIV acts on this acceptor much better than β4Gal-TI, (Fig. 4, C and D), demonstrating that β4Gal-TIV efficiently acts on core 2 branched oligosaccharide regardless of whether or not a hydrophobic aglycon is attached. These results, taken together, support our conclusion that β4Gal-TIV and iGnT cooperatively form poly-N-acetyllactosaminyls on core 2 branched oligosaccharides initiated by C2GnT.

The present study also demonstrated that poly-N-acetyllactosaminylated O-glycans are more efficient than those in N-glycan acceptors when both were synthesized under the same conditions (see Fig. 1A and Fig. 5B). This finding is consistent with the fact that core 2 branched O-glycans are mostly composed of those containing one or two N-acetyllactosamine units, while poly-N-acetyllactosaminyl N-glycans contain three or more N-acetyllactosamine units (4–6, 42–46). Moreover, the amount of poly-N-acetyllactosaminylated O-glycans is much less than that in N-glycans when N-glycans and O-glycans were analyzed in the same lamp molecules (55) or the same CHO cells (46, 48, 55). The present study also demonstrated that β4Gal-TIV, but not β4Gal-TI, drastically reduces its efficiency as acceptors become longer (Fig. 6, Table I). β4Gal-TIV was also shown to act less efficiently on longer lacto-series glycolipids than shorter ones (33). These results, combined together, indicate that the intrinsic nature of β4Gal-TIV is a likely cause for shorter poly-N-acetyllactosaminyls in core 2 branched oligosaccharides.

DISCUSSION

In the present study, we found that β4Gal-TI, abundantly present in milk, cannot efficiently add a galactose residue to a core 2 branched oligosaccharide, GlcNAcβ1→6Manα1→6Manβ→octyl (open circle), and GlcNAcβ1→4Galβ1→4GlcNAcβ1→6Mana1→6Manβ→octyl (closed circle), and GlcNAcβ1→4Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→4Galβ1→6Manα1→6Manβ→octyl (closed square) were incubated with β4Gal-TI (A) or β4Gal-TIV (B) and 5 μmol UDP-[3H]Gal.

Fig. 6. Dependence of β4Gal-TI (A) and β4Gal-TIV (B) on the concentration of acceptors containing different sizes of N-acetyllactosamine repeats. A GlcNAcβ1→6Manα1→6Manβ→octyl (closed circle), GlcNAcβ1→3Galβ1→4GlcNAcβ1→6Manα1→6Manβ→octyl (open circle), and GlcNAcβ1→4Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→6Manα1→6Manβ→octyl (closed square) were incubated with β4Gal-TI (A) or β4Gal-TIV (B) and 5 μmol UDP-[3H]Gal.

After cloning β4Gal-TI from bovine and human milk (50–52), β4Gal-TI has been the only β4Gal-T recognized until recently. The presence of additional members of β4Gal-T was, however, suggested from the knockout of the β4Gal-TI gene in mouse, since the mice deficient in β4Gal-TI survived during embryonic development (53). More recent accumulation in the expressed sequence tag data base, PCR homology cloning, and purification and cloning of lactosylceramide synthase allowed several laboratories to isolate novel members of the β4Gal-T family, β4Gal-TII to β4Gal-TVI (29, 31–33, 49). There appear to be overlapping but different acceptor specificities in β4Gal-TI, -TII, and -TIII, and all of them are capable of adding a galactose residue to form N-acetyllactosamine in both glycoproteins and glycolipids. Except for β4Gal-TIV, which is mainly responsible for lactosylceramide synthesis from glucosylceramide, however, the roles of the novel β4Gal-Ts have been elusive. For example, β4Gal-TIV, which is remotely related to β4Gal-TI, was reported to be inactive toward a glycolipid or glycoprotein acceptor (31).

To our knowledge, the present study is the first report among novel members of β4Gal-Ts that a particular β4Gal-T, β4Gal-TIV, is exclusively responsible for forming specific structures. β4Gal-TIV is the most recently added member of the β4Gal-T gene family (33). In the present study, we demonstrated that β4Gal-TIV is very efficient in adding galactose to the core 2 branched oligosaccharide but inefficient in adding to N-glycan-related acceptors such as GlcNAcβ1→6Manα1→6Manβ→octyl. This finding is consistent with the previous report that β4Gal-TIV adds very little galactose to asialoagalactotransferrin, which contains only N-glycans (33). Our results are also consistent with the report that β4Gal-TIV acts inefficiently on asialoagalactofetuin, since O-glycans in fetuin lack core 2 branches (54). It was shown in the previous report that β4Gal-TIV can act with reasonable efficiency on glycolipid acceptors (33). We were thus concerned about whether the hydrophobic nature of the aglycon, p-nitrophenol in acceptors used, might affect the enzymatic reactions. We thus used a core 2 branched acceptor without an aglycon and found that β4Gal-TIV acts on this acceptor much better than β4Gal-TI, (Fig. 4, C and D), demonstrating that β4Gal-TIV efficiently acts on core 2 branched oligosaccharide regardless of whether or not a hydrophobic aglycon is attached. These results, taken together, support our conclusion that β4Gal-TIV and iGnT cooperatively form poly-N-acetyllactosaminyls on core 2 branched oligosaccharides initiated by C2GnT.

The formation of core 2 branched oligosaccharides has been found to be critical in many biological processes, as described in the Introduction. Further studies for understanding the biosynthesis of core 2 branches and its physiological roles of core 2 branched oligosaccharides.

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