Poly-N-acetyllactosamine Synthesis in Branched N-Glycans Is Controlled by Complemental Branch Specificity of i-Extension Enzyme and β1,4-Galactosyltransferase I*

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Poly-N-acetyllactosamine is a unique carbohydrate that can carry various functional oligosaccharides, such as sialyl Lewis X. It has been shown that the amount of poly-N-acetyllactosamines is increased in N-glycans, when they contain Galβ1→4GlcNAcβ1→6Galβ1→4GlcNAcβ1→2Manα1→6 branched structure. To determine how this increased synthesis of poly-N-acetyllactosamines takes place, the branched acceptor was incubated with a mixture of i-extension enzyme (iGnT) and β1,4-galactosyltransferase I (β4Gal-TI). First, N-acetyllactosamine repeats were more readily added to the branched acceptor than the summation of poly-N-acetyllactosamines formed individually on each unbranched acceptor. Surprisingly, poly-N-acetyllactosamine was more efficiently formed on Galβ1→4GlcNAcβ1→2Manα1→R side chain than in Galβ1→4GlcNAcβ1→6Manα1→R, due to preferential action of iGnT on Galβ1→4GlcNAcβ1→2Manα1→R side chain. On the other hand, galactosylation was much more efficient on β1,6-linked GlcNAc than β1,2-linked GlcNAc, preferentially forming Galβ1→4GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6Manβ→R. Starting with this preformed acceptor, N-acetyllactosamine repeats were added almost equally to Galβ1→4GlcNAcβ1→6Manα1→R and Galβ1→4GlcNAcβ1→2Manα1→R side chains. Taken together, these results indicate that the complemental branch specificity of iGnT and β4Gal-TI leads to efficient and equal addition of N-acetyllactosamine repeats on both side chains of GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6Manβ→R structure, which is consistent with the structures found in nature. The results also suggest that the addition of Galβ1→4GlcNAcβ1→6 side chain on Galβ1→4GlcNAcβ1→2Manα1→R side chain converts the acceptor to one that is much more favorable for iGnT and β4Gal-TI.

In human granulocytes, monocytes, and certain T lymphocytes, on the other hand, poly-N-acetyllactosamines contain Leα,1 Galβ1→4(Fucα1→3GlcNAc→R, and sialyl Leα, NeuNAcα2→3Galβ1→4(Fucα1→3GlcNAc→R (6–9). Sialyl Leα and its sulfated forms are ligands for E-, P- and L-selectin (10–12). During inflammation, E- and P-selectin expressed on activated endothelial cells bind to sialyl Leα oligosaccharides present on granulocytes, and such initial binding leads to the extravasation of granulocytes (10–12). L-selectin on lymphocytes, on the other hand, recognizes sulfated sialyl Leα expressed in high endothelial venules of blood vessels (13–15). This L-selectin-counter-receptor interaction enables lymphocytes to migrate into lymphoid system, allowing lymphocytes to circulate fully in the body.

Poly-N-acetyllactosamines are attached to N-glycans (3–7, 16–18), O-glycans (8, 9, 19), and glycolipids (20–22). Poly-N-acetyllactosamines are synthesized through alternate actions of β1,3-N-acetylgalactosaminyltransferase, i-extension enzyme (iGnT), and β1,4-galactosyltransferase (β4Gal-T). In N-glycans, poly-N-acetyllactosamines are found more often in tetraantennary and triantennary N-glycans that contain a side chain linked to α,1,6-linked mannose through a GlcNAcβ1→6 linkage in GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6Manβ→R (23–27). This side chain is formed by N-acetylgalactosaminyltransferase V (GnTV). Significantly, the amount of GnTV is increased in various tumors including colonic carcinoma cells and those transformed with oncogenes (23–27). Overexpression of GnTV in cultured cells was reported to result in acquiring the capability of growth in soft agar and tumor formation after subcutaneous injection of the transfected cells (28).

The increased expression of sialyl Leα apparently takes place in those tumor cells when α,1,3-fucosyltransferase is also present. In fact, highly metastatic colonic carcinoma cells express more sialyl Leα in poly-N-acetyllactosamines than poorly metastatic counterparts (27). Moreover, our recent studies demonstrated that B16 mouse melanoma cells produced many more lung tumor foci after the cells were transfected with α,1,3-fucosyltransferase to form sialyl Leα in poly-N-acetyllactosamines (29). These results, as a whole, indicate that the formation of poly-N-acetyllactosamine plays a critical role for carbohydrate recognition in cell-cell interaction.

Previously, we have demonstrated that poly-N-acetyllactosamines in core 2 branched O-glycans are synthesized through iGnT and a newly discovered member of β1,4-galactosyltransferase, β4Gal-TIV (30). We have also found that β4Gal-TI, iGnT, and β1,4-galactosyltransferase are involved in the synthesis

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The abbreviations used are: Leα, Lewisα; GnTV, N-acetylgalactosaminyltransferase V; iGnT, i-extension β1,3-N-acetylgalactosaminyltransferase; β4Gal-T, β1,4-galactosyltransferase; HPLC, high performance liquid chromatography.
of I-branched poly-N-acetyllactosaminies (31). In the same study, we found that the addition of N-acetyllactosaminie repeats to linear poly-N-acetyllactosaminies is preferred over the extension of N-acetyllactosamine to I-branaches, mainly because the first galactosylation of a GlcNAcβ1→6 branch is inefficient. These studies thus demonstrate intricate interaction between a specific glycosyltransferase and an acceptor molecule, which largely contributes to the control of poly-N-acetyllactosaminie synthesis. In these studies, we utilized molecular tools that have recently become available, such as cDNAs encoding i-extension enzyme (32), novel members of β4Gal-T (33), and I-branaching enzyme (34).

These results prompted us in the present study to determine how poly-N-acetyllactosaminie is formed in branched N-glycans. Our results demonstrated that the addition of GlcNAcβ1→6Manо→R side chain renders the resultant GlcNAcβ1→6GlcNAcβ1→2Manα1→6Manβ→R an extremely efficient acceptor for β4Gal-Ti and i-extension enzyme. Moreover, we found that the i-extension enzyme prefers Galβ1→4GlcNAcβ1→2Manα1→6Manβ→R side chain over Galβ1→4 GlcNAcβ1→6Manо→R side chain, whereas the opposite is true for β4Gal-TI. Such complementary effects apparently result in forming similar size and abundance of poly-N-acetyllactosaminies in both chains of GlcNAcβ1→6 (GlcNAcβ1→2)-Manα→R structures.

EXPERIMENTAL PROCEDURES

Isolation and Expression of cDNA Encoding iGnT—cDNA encoding iGnT was cloned into pcDNA3.1, resulting in pcDNA3.1-I-GnT as described previously (32). pcDNA-I, harboring cDNA encoding a signal sequence and an IgG binding domain of Staphylococcus aureus protein A, was constructed as described before (35). The catalytic domain of iGnT was cloned into this vector, resulting in pcDNA-I-GnT (32). pcDNA-I and pcDNA-I-GnT were separately transfected with LipofectAMINE Plus (Life Technologies, Inc.) into COS-1 cells as described previously (36). The chimeric enzyme released into serum-free preparation (Sigma) was directly used as 1000-fold by a Centricon 10 concentrator (Amicon) and directly used after 12 h, additional UDP-galactose (1.5 mM) was added, and incubation was continued for another 12 h. TLC of the products indicated absence of the starting material. Compounds 11 and 12 were isolated as described above and determined to have specific activities of ~8.5 Ci/mol.

Compound 1 (3 mM) was treated with jack bean β-N-acetylgalactosaminidase (4 units) in 50 mM sodium acetate buffer, pH 5.5, at 37 °C for 3 days, resulting in the tetrasaccharide Galβ1→4GlcNAcβ1→2 Manα1→6Manβ→octyl (compound 13). Similarly, compound 1 (5 mM) was treated with Escherichia coli β-galactosidase (20 units) in 50 mM Tris-HCl buffer, pH 7.4, at 37 °C overnight, resulting in the tetrasaccharide GlcNAcβ1→6GlcNAcβ1→2Manα1→6Manβ→octyl (compound 14). Isolation, purification, and characterization of these compounds was performed as described above. Detailed procedures of these syntheses will be published elsewhere.

Partial 1H NMR results (300 MHz, D2O) (compounds 1, 2, 13, and 14) are as follows. Compound 1: 6.49 (s, H-2), 4.65 (s, H-1), 4.63 (d, J = 7.8 Hz), 4.48 (d, J = 7.8 Hz), 4.44 (d, J = 7.8 Hz), 4.38 (d, J = 7.8 Hz), 4.18 (d, J = 7.8 Hz), 2.09, 2.03 (2 s, 6H, NHAc). Compound 2: 6.45 (s, H-2), 2.95 (2 s, 6H, NHAc). Compound 3: 4.88 (s, H-1), 4.66 (s, H-1), 4.60–4.55 (m, 2H), 4.47 (d, J = 8.3 Hz, 1H), 2.06, 2.03 (2 s, 6H, NHAc). Compound 13: 4.94 (s, H-1), 4.67 (s, H-1), 4.63–4.60 (m, 1H), 4.48 (d, J = 7.8 Hz, 1H), 2.06 (s, 3H, NHAc). Compound 14: 4.87 (s, H-1), 4.66 (s, H-1), 4.58 (d, J = 8.1 Hz, 1H), 4.54 (d, J = 8.1 Hz, 1H), 2.05, 2.03 (2 s, 6H, NHAc).

The Addition of N-Acetylgalactosamine by iGnT—To assay the transfer of N-acetylgalactosamine residues by the iGnT, the reaction mixture was exactly the same as described previously (30, 32). The incubation mixture was applied to a C18-reverse phase Sep-Pak cartridge column (Waters) and the product was eluted as described previously (30). The product was then analyzed by HPLC using NH2-bonded silica column (Varian Micropak AX-5) as described previously (30). The radioactivity of aliquots in the effluent was determined. The iGnT was also incubated with [3H]Galβ1→4GlcNAcβ1→6Manα1→6Manβ→octyl or Galβ1→4GlcNAcβ1→6 (GlcNAcβ1→2Manα1→6Manβ→octyl) in these experiments, nonradioactive 5 mM UDP-GlcNAc was used. In all of the above reactions, the reaction mixture was incubated for 10 h to analyze the products or for 1 h to obtain kinetic parameters.

Poly-N-acetyllactosaminie Synthesis by iGnT and βGal-T To assay poly-N-acetyllactosaminie formation, 0.5 mM acceptor was incubated with βGal-T (760.0 mmol/m1) and iGnT (380.0 mmol/m1) under the conditions described previously (30, 32).

When [3H]Galβ1→4GlcNAcβ1→6Manα1→6Manβ→octyl (see above) and Galβ1→4GlcNAcβ1→6 (GlcNAcβ1→2Manα1→6Manβ→octyl) (compound 12, see above) were used as acceptors, nonradioactive donor substrates were used.

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The products were purified by HPLC using the same NH$_2$-bonded silica column as described above. A peak containing one N-acetyllactosamine repeat was digested with diplococcal $\beta$-galactosidase (41), and the digest was purified by a Sep-Pak column and then analyzed by HPLC as described above. A peak containing two N-acetyllactosamine repeats was sequentially digested with diplococcal $\beta$-galactosidase, jack bean $\beta$-N-acetylglucosaminidase, and diplococcal $\beta$-galactosidase. After each digestion, the digest was analyzed by HPLC using the same NH$_2$-bonded column as described above. Aliquots were taken for determining radioactivity, obtaining the ratio of the radioactivity in the product and starting material.

As a third experiment, 0.5 mM Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6GlcNAc$\beta$$\rightarrow$2Man$_1$$\rightarrow$6Man$_1$-octyl or 0.5 mM GlcNAc$\beta$$\rightarrow$6Gal$_1$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2Man$_1$$\rightarrow$6Man$_1$-octyl was incubated with $\beta$Gal-TI (152.0 nmol/h/ml), iGnT (38.0 or 76.0 nmol/h/ml), 0.5 mM UDP-[3H]Gal in 50 mM NaCl and 10 mM each of GlcNAc-1,5-lactone and Gal-1,5-lactone. After incubation at 37°C for 4 h, the reaction products were purified by a Sep-Pak column and subjected to HPLC as described above. In these experiments, the incubation conditions were first determined where only one N-acetyllactosamin unit can be added to Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$3Gal$\beta$$\rightarrow$Glc. In addition, $\beta$Gal-TI was 4-fold in excess over iGnT, the same ratio as in HL-60 cells (42). In certain experiments, $\beta$Gal-TI was 2-fold in excess over iGnT.

**Analysis of Products by Endo-$\beta$-Galactosidase Digestion—** Products were digested with Escherichia freundii endo-$\beta$-galactosidase for 18 h at 37°C (43). The digestion conditions used allowed the cleavage of galactose linkage, where no $\beta$-1,6-linked N-acetyllactosamine is attached (2, 43). The digests were subjected to HPLC using AX-5 column.

**RESULTS**

Addition of N-Acetylgalactosamine to N-Glycan Acceptor by i-Extension Enzyme—To determine whether iGnT has a preference for the addition of N-acetylgalactosamine to one of N-glycan acceptors, Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6Man$_1$$\rightarrow$R, Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2Man$_1$$\rightarrow$R, and Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6(Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2)Man$_2$$\rightarrow$R were incubated with iGnT. First, iGnT acted in almost identical efficiency on Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6Man$_1$$\rightarrow$R and Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2Man$_2$$\rightarrow$R (Figs. 1A and 2, A and B), indicating that the enzyme does not prefer one acceptor over the other. Second, the branched acceptor has a better affinity and higher $V_{\text{max}}$ for addition of one N-acetylgalactosamine (Fig. 1A and Table I). When the products from the 10-h incubation of the branched acceptor was analyzed, the majority of the products contained only one N-acetylgalactosamine residue, and a more 5.7% (in molar ratio) of the whole products contained two N-acetylgalactosamine residues (Fig. 2C).

**FIG. 1.** Dependence of iGnT and $\beta$Gal-Ts on the concentration of linear and branched N-glycan acceptors. A, Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2Man$_1$$\rightarrow$6Man$_1$-octyl (●), Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2Man$_1$$\rightarrow$6Man$_1$-octyl (○), or Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6Man$_1$$\rightarrow$6Man$_1$-octyl (●) of various concentrations was incubated with iGnT for 1 h. B, GlcNAc$\beta$$\rightarrow$2Man$_1$$\rightarrow$6Man$_1$-octyl of various concentrations was incubated with $\beta$Gal-TI (●), $\beta$TI (○), $\beta$TV (□), and $\beta$TV (■) for 1 h. The same amount of the enzyme, 38.0 nmol/h/ml determined using 0.5 mM Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$γ-nitrophenol (A) or 0.5 mM GlcNAc$\beta$$\rightarrow$γ-nitrophenol (B), was present in those experiments.

**TABLE I**

| Substrate | $K_m$ (mm) | $V_{\text{max}}$ (nmol/h/ml) |
|-----------|-----------|-------------------------------|
| Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6Man$_1$-6Man$_1$-octyl | 1.19 | 100* |
| Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2Man$_1$-6Man$_1$-octyl | 1.20 | 105 |
| Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6Man$_1$-6Man$_1$ | 0.81 | 155 |
| GlcNAc$\beta$$\rightarrow$2Man$_1$-6Man$_1$-octyl | 0.77 | 75 |

* Relative values. $V_{\text{max}}$ of iGnT is compared to the $V_{\text{max}}$ (97.8 nmol/h/ml) obtained using Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6Man$_1$-6Man$_1$-octyl as an acceptor.

**FIG. 2.** Analysis of the products after 10 h-incubation of N-glycan acceptors with iGnT. A–C, HPLC analysis of the iGnT products derived from Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6Man$_1$$→$R (A), Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2Man$_1$$→$R (B), and Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2Man$_2$$→$R (C). Numbers indicate the relative ratio of incorporated radioactivity. Peaks at Fraction 34 and 37 in C correspond to the products containing one and two GlcNAc residues, respectively. D, HPLC analysis of the iGnT product from [3H]Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6(Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2)Man$_2$$→$R. Peaks at Fractions 31 and 35 correspond to the starting material and the product containing one GlcNAc residue, respectively. E, HPLC analysis of the product substituted with one N-acetylgalactosamine, shown in D (Fraction 35) after endo-$\beta$-galactosidase digestion (solid line). F, HPLC analysis after endo-$\beta$-galactosidase digestion of the product substituted with one N-acetylgalactosamine (solid line), which was derived from Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6[3H]Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2Man$_1$$→$R. In E and F, the mono-N-acetylgalactosaminylated products are shown as dotted lines. Numbers indicate the relative ratio of these oligosaccharides.

To determine which side chain is substituted with N-acetylgalactosamine, [3H]Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$6Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2Man$_2$$→$R was first synthesized with $\beta$Gal-TI and UDP-[3H]Gal from GlcNAc$\beta$$\rightarrow$6Gal$\beta$$\rightarrow$4GlcNAc$\beta$$\rightarrow$2Man$_2$$→$R. The radioactively labeled acceptor was then incubated with iGnT and unlabeled UDP-GlcNAc. As shown in Fig. 2D, the product containing one GlcNAc addition (Fraction 35) was obtained together with the starting material (Fraction 31). The product substituted with one N-acetylgalactosamine was digested with endo-$\beta$-galactosidase, and the digest was...
subjected to the same HPLC. Seventy-five % of the radioactivity was recovered as [3H]Galβ1→4GlcNAcβ1→6(GlcNAcβ1→2)Manα1→R (Fig. 2E), indicating that 75% of the products was [3H]Galβ1→4GlcNAcβ1→6(GlcNAcβ1→3)Galβ1→4GlcNAcβ1→2Manα1→R, whereas 25% was GlcNAcβ1→3[3H]Galβ1→4GlcNAcβ1→6Galβ1→4GlcNAcβ1→2Manα1→R (see Fig. 3A).

To corroborate the above results, Galβ1→4GlcNAcβ1→6[3H]Galβ1→4GlcNAcβ1→2Manα1→R was synthesized from Galβ1→4GlcNAcβ1→6(GlcNAcβ1→2)Manα1→R, and the resultant acceptor was incubated with iGnT and UDP-GlcNAc. The monosubstituted product obtained was then digested with endo-β-galactosidase, producing GlcNAcβ1→6[3H]Galβ1→4GlcNAcβ1→2Manα1→R with 26% yield (Fig. 2F). The results thus indicate that 26% of the products was GlcNAcβ1→3Galβ1→4GlcNAcβ1→6[3H]Galβ1→4GlcNAcβ1→2Manα1→R, whereas 74% was Galβ1→4GlcNAcβ1→6GlcNAcβ1→4[3H]Galβ1→4GlcNAcβ1→2Manα1→R (Fig. 2F, see also Fig. 3B).

These results, taken together, indicate that iGnT preferentially acts on Galβ1→4GlcNAcβ1→2Manα1→R side chain over Galβ1→4GlcNAcβ1→6Manα1→R in an approximate ratio of 3:1 under the incubation conditions used.

**Poly-N-acetyllactosamine Synthesis on Unbranched or Branched Acceptor**—As shown previously (30), among different members of β4Gal-Ts β4Gal-TI was most efficient in adding galactose to GlcNAcβ1→6Manα1→6Manβ1→octyl. Similarly, we found in the present study that β4Gal-TI is the most efficient in adding a galactose to GlcNAcβ1→2Manα1→6Manβ1→octyl (Fig. 1B; see also Table II).

To determine how N-acetyllactosamine repeats are added to two different side chains, Galβ1→4GlcNAcβ1→6Manα1→R or Galβ1→4GlcNAcβ1→2Manα1→R was incubated with iGnT, β4Gal-TI, UDP-[3H]Gal and UDP-[3H]GlcNAc. Fig. 4, A and B, illustrates that poly-N-acetyllactosamines were almost equally formed from these two acceptors. We then incubated a branched acceptor, Galβ1→4GlcNAcβ1→6Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→octyl with iGnT, β4Gal-TI, and radioactive donor substrates. First, the branched acceptor incorporated 8.4 times more [3H]Gal and [3H]GlcNAc than the unbranched acceptors when incubated under the same conditions (Fig. 4C). The results also demonstrated that the branched acceptor contained more N-acetyllactosamine units, and the product containing three N-acetyllactosamine units constituted the major product (Fig. 4C). In contrast, the major product obtained from the unbranched acceptors contained one N-acetyllactosamine unit (Fig. 4, A and B). These results indicate that the addition of Galβ1→4GlcNAcβ1→6 branch to Galβ1→4GlcNAcβ1→2Manα1→R in the acceptor has a synergistic effect on the branched acceptor, forming many more N-acetyllactosamine repeats than a summation of N-acetyllactosamine repeats formed when each branch was individually assayed. Moreover, such an effect extends to the formation of longer poly-N-acetyllactosamines.

**Poly-N-acetyllactosamine Is Preferentially Added to Galβ1→4GlcNAcβ1→2 Side Chain on Galβ1→4GlcNAcβ1→2Galα1→4GlcNAca1→6Manα1→R Acceptor**—To determine how each side chain of the branched acceptor was elongated with N-acetyllactosamine repeats, [3H]Galβ1→4GlcNAcβ1→6Galβ1→4GlcNAcβ1→2Manα1→R, synthesized as described above, was incubated with iGnT, β4Gal-TI, and nonradioactive donor substrates. The products obtained exhibited an elution profile similar to that obtained in the above experiments (Fig. 4D). The difference between Fig. 4C and Fig. 4D was due to the fact that larger products were more visible in Fig. 4C because those products contained a greater amount of [3H]GlcNAc and [3H]Gal than smaller products.

The product containing one N-acetyllactosamine repeat (Fig. 4D, peak 1) was digested with exo-β-galactosidase. As shown in Fig. 5A, 28.6% of the starting product was recovered as GlcNAcβ1→3[3H]Galβ1→4GlcNAcβ1→6(GlcNAcβ1→2)Manα1→R. The results indicate that 28.6% of the products was Galβ1→4GlcNAcβ1→6Galβ1→4GlcNAcβ1→2Manα1→R, whereas 71.4% was [3H]Galβ1→4GlcNAcβ1→6Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→2Manα1→R, which produced a nonradioactive compound after β-galactosidase treatments.

Similarly, the products containing two N-acetyllactosamine repeats (Fig. 4D, peak 2) were sequentially digested with β-galactosidase, β-N-acetylgalactosaminidase, and β-galactosidase. After first β-galactosidase digestion, 35.2% of the products was recovered as radioactive oligosaccharides, indicating that 48.8% of the starting products was [3H]Galβ1→4GlcNAcβ1→6(GlcNAcβ1→3), GlcNAcβ1→2Manα1→R (Fig. 5B, see also Fig. 6B, middle). The additional digestion of the above β-galactosidase digest with β-N-acetylgalactosaminidase resulted in no release of [3H]galactose as expected (Fig. 5C). The above digested products were finally digested again with β-galactosidase, which recovered 74.1% of the radioactivity.
Arrows indicate where galactose is added.

| Substrate | β4Gal-TI | β4Gal-TII | β4Gal-TIII | β4Gal-TIV | β4Gal-TV |
|-----------|----------|-----------|------------|-----------|----------|
| GlcNAcβ1–6Manα1–6Manβ–octyl | 100% | 30% | 63% | 43% | 63% |
| GlcNAcβ1–2Manα1–6Manβ–octyl | 96% | 32% | 70% | 46% | 65% |

* Relative values. V_{max} for β4Gal-Ts is compared to the V_{max} (118.3 nmol/h/ml) obtained for β4Gal-TI using GlcNAcβ1–6Manα1–6Manβ–octyl as an acceptor.

**Table II**

Kinetic properties of β4Gal-Ts

Arrows indicate where galactose is added.

![HPLC analysis of the products after incubation of N-glycan acceptors with iGnT and β4Gal-TI.](image)

![Fraction Number vs CPM](image)

The results shown in Fig. 5, E–H, are mirror images of those described in Fig. 5, A–D. The results thus indicate that the products with two N-acetyllactosamine repeats were a mixture of (Gal β1→4GlcNAcβ1→3)Galβ1→4GlcNAcβ1→6(3H)Galβ1→4GlcNAcβ1→2Manα→R (26.4%), Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→3)2(3H)Galβ1→4GlcNAcβ1→2Manα→R (63.6%), and Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→2Manα→R (10%) (see Fig. 6D).

These results, taken together, indicate that preferential addition of N-acetylgalactosamine by iGnT to Galβ1→4GlcNAcβ1→2Manα→R side chain leads into preferential formation of poly-N-acetyllactosamine in Galβ1→4GlcNAcβ1→2Manα→R branch.

Galactose Is Preferentially Added to GlcNAcβ1–6Manα→R Branch Over GlcNAcβ1–2Manα→R Branch—The next question we asked is how the first galactosylation influences the formation of poly-N-acetyllactosamines. GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα→R or Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα→R was incubated with βGal-TI and UDP(3H)Gal. As shown in Fig. 7A, β4Gal-TI adds a galactose to GlcNAcβ1→6Manα→R branch much more efficiently than the GlcNAcβ1→2Manα→R branch. The kinetic efficiency (V_{max}/K_{m}) exhibited a 2.5-fold difference between these two acceptors (Table III).

The above results indicate that galactose is preferentially added to GlcNAcβ1–6Manα→R branch, forming Galβ1→4GlcNAcβ1→6Manα→R branch. We then tested how N-acetyllactosamine repeats are added on these preformed acceptors. First, the addition of N-acetylgalactosamine by iGnT was found to be almost equal between Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα→R and GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα→R (Figs. 7B and Table I).

Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα→R was then incubated with iGnT, β4Gal-TI, UDP(3H)GlcNAc, and UDP(3H)Gal under conditions where only one N-acetyllactosamine extension takes place. As products, GlcNAcβ1→6(Galβ1→4GlcNAcβ1→3)Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα→R and Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→3)Galβ1→4GlcNAcβ1→2Manα→R were expected (Figs. 8, A and B, dotted line). When these products are digested by endo-β-galactosidase, a radioactive product would be derived only from (3H)GlcNAcβ1→3Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα→R. Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα→R and Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→3)Galβ1→4GlcNAcβ1→2Manα→R would lose radioactivity after endo-β-galactosidase digestion (endo-β-galactosidase-susceptible galactose is underlined in both structures) (see Fig. 9A).

First, the acceptor was incubated with iGnT and β4Gal-TI in a ratio of 1:4, as done for all the other experiments. The products obtained from this reaction resulted in GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα→R with a 1.0:9.3

![Poly-N-acetyllactosamine Synthesis in Branched N-Glycans](image)
yield after endo-β-galactosidase digestion. The results indicate that the molar ratio between Galβ1→4GlcNAcβ1→6[3H]Galβ1→4GlcNAcβ1→2Manα-R and the whole products was 1.0:3.1, because each individual product contained three radioactive sugars (Fig. 8A; see also Fig. 9A). This can be translated into the conclusion that the N-acetyllactosamine repeat was formed on Galβ1→4GlcNAcβ1→6Manα-R and GlcNAc-β1→2Manα-R in the acceptor in a ratio of 1:2:1.

When the same acceptor was incubated with increased amount of iGnT, and the ratio of iGnT and β4Gal-TI was 1:2, 1:4:7:2 of the radioactivity was recovered as GlcNAc-β1→6[3H]Galβ1→4GlcNAcβ1→2Manα-R after endo-β-galactosidase digestion (Fig. 8B). The results indicate that N-acetyllactosamine repeat was added to Galβ1→4GlcNAcβ1→6Man branch 1.4 times more than to GlcNAcβ1→2Man branch. These results indicate that both branches were almost equally elongated by N-acetyllactosamine repeats when iGnT and β4Gal-TI was in a ratio of 1:2 under the incubation conditions employed (Fig. 9A).

In parallel, GlcNAcβ1→6Galβ1→4GlcNAcβ1→2Manα-1→6Manβ1→octyl was incubated with iGnT, β4Gal-TI, and radioactive donor substrates under the same conditions described above. In these experiments, [3H]Galβ1→4GlcNAcβ1→2Manα-R should be produced only from [3H]Galβ1→4GlcNAcβ1→6[3H]Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→2Manα-R after endo-β-galactosidase digestion (the endo-β-galactosidase-susceptible galactose is underlined; see Fig. 9B). As shown in Fig. 8, C and D, the ratio between the oligosaccharide synthesized and product after endo-β-galactosidase digestion was found to be 10.5:2.5 in both conditions. These results indicate that the products contained [3H]Galβ1→4GlcNAcβ1→6[3H]Galβ1→4[3H]GlcNAcβ1→3Galβ1→4GlcNAcβ1→2Manα-R and [3H]Galβ1→4[3H]GlcNAcβ1→3[3H]Galβ1→4GlcNAcβ1→6Galβ1→4GlcNAcβ1→2Manα-R in a ratio of 2.5:1.0. The results indicate that poly-N-acetyllactosamine extension preferentially takes place on Galβ1→4GlcNAcβ1→2Manα-R side chain when the reaction was started from GlcNAcβ1→6Galβ1→4GlcNAcβ1→2Manα-R, regardless of the ratio of iGnT and β4Gal-TI.

As shown above, galactosylation takes place preferentially on β1,6-linked GlcNAc in GlcNAcβ1→6(GlcNAcβ1→2Manα-1→6Manβ1→octyl. These combined results thus indicate that poly-N-acetyllactosamine extension almost equally takes place on both GlcNAcβ1→6Man and GlcNAcβ1→2Man side chains after the formation of Galβ1→4GlcNAcβ1→6(GlcNAcβ1→2Manα-1→6Manβ-R.

**Discussion**

The present study demonstrates that Galβ1→4GlcNAcβ1→2Manα-R antenna is extended by N-acetyllactosamine repeats as much as does Galβ1→4GlcNAcβ1→6Manα-R antenna (Figs. 1 and 2). When both galactosylated side chains are present in a branched acceptor, Galβ1→4GlcNAcβ1→2Manα-R is utilized more efficiently to add N-acetyllactosamine repeats than Galβ1→4GlcNAcβ1→6Manα-R (Figs. 4–6). This is mainly because iGnT preferentially acts on Galβ1→4GlcNAcβ1→2Manα-R side chain (Figs. 2 and 3). Similarly, it was reported that iGnT from Novikoff hepatoma acts 1.6 times more efficiently on Galβ1→4GlcNAcβ1→2Manα-R side chain than on Galβ1→4GlcNAcβ1→6Manα-R side chain in a branched acceptor (44). The results suggest that iGnT present in Novikoff hepatoma is probably the same as the cloned iGnT used in the present study.

Moreover, the amount of poly-N-acetyllactosamine formed in the branched acceptor was much more than the summation of two separate reactions using either one of these side chains (Fig. 4). These results strongly suggest that addition of Galβ1→4GlcNAcβ1→6 side chain on Galβ1→4GlcNAcβ1→2Manα-R side chain, or vice versa, must change the conformation of Galβ1→4GlcNAcβ1→2Manα-R and possibly that of Galβ1→4GlcNAcβ1→6Manα-R. As a result, both side chains become more favorable for iGnT and β4Gal-TI to act.
in Fig. 5, [50x371]E
de note the relative molar ratio of the products formed. The results shown in Fig. 5, [50x170]A and UDP-[3H]Gal ([50x94]
result indicates that the action of GnTV restricts the confor-
amation takes place on
GlcNAc [50x136]
This enhancement can be observed even when first galactosyla-
tion takes place on
GlcNAc [50x146]
chemical synthesis may also bring some conformational change
in both side chains, which is favorable for the actions by iGnT
and [β4Gal-TI].

It is rather striking that Galβ1→4GlcNAcβ1→2Man
α1→6Manβ→R is as good as Galβ1→4GlcNAcβ1→6Man
α1→6Manβ→R as an acceptor for iGnT (Figs. 1 and 2 and
Table I). The results obtained in the present study are, how-
ever, consistent with the structural data on human red cell
band 3 (18, 47). Human red cell band 3 does not contain
Galβ1→4GlcNAcβ1→6Manα1→6Manβ→R side chain yet

This enhancement can be observed even when first galactosyla-
tion takes place on GlcNAcβ1→6(GlcNAcβ1→2)Man
α1→6Manβ→octyl (see Tables II and III). In this context, it is
noteworthy that GnTV adds N-acetylglucosamine only when
Manα1→6Man is in a gauche-gauche conformation (45). This
result indicates that the action of GnTV restricts the confor-
manation of resultant branched oligosaccharide, GlcNAcβ1→6(Glc-
NAcβ1→2)Manα1→6Manβ→octyl and their sequential enzymatic digestion products are shown. C and D, the products derived from Galβ1→4GlcNAc-
β1→6[3H]Galβ1→4GlcNAcβ1→2Manα1→6Manβ→octyl were analyzed after sequential exoglycosidase digestions. Numbers in parentheses
denote the relative molar ratio of the products formed. The results shown in Fig. 5, 3A–D, correspond to Fig. 6, A and B, whereas the results shown in
Fig. 5, 3E–H, correspond to Fig. 6, C and D. Man ( ), GlcNAc ( ), galactose ( ), and radioactive galactose (°) are denoted.

**TABLE III**
Kinetic properties of β4Gal-TI

| Substrate | $K_m$ (mM) | $V_{max}$ (μmol/h/ml) |
|-----------|------------|-----------------------|
| -GlcNAcβ1 | 2.29       | 347°                  |
| Galβ1→4GlcNAcβ1→2Manα1→6Manβ→octyl | 1.41       | 85                    |
| Galβ1→4GlcNAcβ1 | 0.33 | 197°                  |

° Relative values. $V_{max}$ of β4-Gal-TI is compared to the $V_{max}$ (118.3
nmol/h/ml) obtained using GlcNAcβ1→6Manα1→6Manβ→octyl as an
acceptor.

Only one galactose was added under the incubation conditions used.
However, distribution between these two side chains was not deter-
mained because Galβ1→4GlcNAcβ1→2Manα1→6Manβ→octyl and Galβ1→
4GlcNAcβ1→6Manα1→6Manβ→octyl, which were obtained after β-N-
acetylglucosaminidase treatment of products, could not be separated in
various chromatographic conditions.

Fig. 6. Schematic representation of the poly-N-acetyllactosaminyl products derived from asymmetrically labeled acceptors. A and
B, the products containing one (A) and two (B) N-acetyllactosamine repeats derived from [3H]Galβ1→4GlcNAcβ1→2Manα1→6Manβ→octyl and their sequential enzymatic digestion products are shown. C and D, the products derived from Galβ1→4GlcNAc-
β1→6[3H]Galβ1→4GlcNAcβ1→2Manα1→6Manβ→octyl were analyzed after sequential exoglycosidase digestions. Numbers in parentheses
denote the relative molar ratio of the products formed. The results shown in Fig. 5, A–D, correspond to Fig. 6, A and B, whereas the results shown in
Fig. 5, E–H, correspond to Fig. 6, C and D. Man ( ), GlcNAc ( ), galactose ( ), and radioactive galactose (°) are denoted.

Fig. 7. Dependence of β4Gal-TI or iGnT on the concentration of acceptors. GlcNAcβ1→6Galβ1→4GlcNAcβ1→2Manα1→6Manβ→octyl ( ), Galβ1→4GlcNAcβ1→6GlcNAcβ1→2Manα1→6Manβ→octyl ( ), and GlcNAcβ1→6GlcNAcβ1→2Manα1→6Manβ→octyl ( ) of various concentrations were incubated with β4Gal-TI
and UDP-[3H]Gal (A) or iGnT and UDP-[3H]GlcNAc (B).
contains very extended poly-N-acetyllactosamines. It is also noteworthy that a side chain extending from Manα1→6Manβ→R contains more poly-N-acetyllactosamines than that extending from Manα1→3Manβ→R in human red cell band 3 (18, 47).

It is assumed that GnT adds N-acetylgalactosamine as soon as GlcNAcβ1→2Manα1→6Manβ→R is formed (48). The resultant oligosaccharide, GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6Manβ→R, is most likely an acceptor for β4Gal-TI. The present study demonstrated that GlcNAcβ1→6 residue is much more favored by β4Gal-TI over GlcNAcβ1→2 residue, although iGnT does not have such a preference (Fig. 7). This finding is similar to those obtained on galactosylation of GlcNAcβ1→6(GlcNAcβ1→3)Gal, I-branch precursor structure. In that particular study, almost 95% of the product was Galβ1→4GlcNAcβ1→6(GlcNAcβ1→3)Gal (49). Such a preferential galactosylation of GlcNAcβ1→6 takes place when both branches are terminated with GlcNAc residues.

The present study demonstrated that poly-N-acetyllactosamine extension takes place more efficiently on Galβ1→4GlcNAcβ1→2Manα1→6Manβ→R side chain than Galβ1→4GlcNAcβ1→2Manα1→6Manβ→R side chain when a fully galactosylated branched acceptor was utilized (Figs. 4 and 5). In contrast, poly-N-acetyllactosamine extension took place almost equally between two branches when increased amount of iGnT and Galβ1→4GlcNAcβ1→6(GlcNAcβ1→2)Manα1→R as an acceptor were used (Figs. 8 and 9). The results obtained in the latter experiments are consistent with those obtained on the structural analysis of glycoproteins containing poly-N-acetyllactosamines from granulocytes (6, 7) and glycoproteins, such as human erythropoietin produced in Chinese hamster ovary cells (50–54). In particular, NMR studies of the N-glycans isolated from the recombinant erythropoietin demonstrated that N-acetyllactosamine extension takes place almost equally in Galβ1→4GlcNAcβ1→6Manα1→R and Galβ1→4GlcNAcβ1→2Manα1→R side chains (52-54). These results, as a whole, strongly suggest that the concentration of iGnT relative to that of β4Gal-TI may be more than that estimated from the activities in total cell lysates. Further studies will be of significance to determine whether iGnT is more enriched in narrower compartments of the Golgi than β4Gal-TI (see also Ref. 55).

The above results were obtained most likely due to the following reasons. First, it is almost certain that Galβ1→4GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6Manβ→R is formed before the formation of GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα1→6Manβ→R, considering that β4Gal-TI greatly prefers β1,6-linked GlcNAc over β1,2-linked GlcNAc residue (Fig. 10b). This allows poly-N-acetyllactosamine extension in Galβ1→4GlcNAcβ1→6Man side chain before initiation of poly-N-acetyllactosamine synthesis in GlcNAcβ1→2Man side chain (Fig. 10, B–E). In contrast, iGnT preferentially acts on Galβ1→4GlcNAcβ1→2Manα1→R side chain once this side chain is galactosylated. Such a branch specificity of iGnT compensates the inefficient galactosylation of GlcNAcβ1→2Manα1→R branch, leading into N-acetyllactosamine extension in Galβ1→4GlcNAcβ1→2Manα1→R branch (Fig. 10f). These results indicate that branch specificity of β4Gal-TI and iGnT has the opposite effect on poly-N-acetyllactosamine extension in the GlcNAcβ1→6Manα1→R branch versus the GlcNAcβ1→2Manα1→R branch. Such complemental branch specificities of β4Gal-TI and iGnT is a likely cause for almost equal distribution of poly-N-acetyllactosamine extension in two side chains in nature. Recently, a new member of iGnT was reported (56). However, no studies on branch specificity of this enzyme was carried out, and it is not known whether this additional iGnT may be responsible for poly-N-acetyllactosamine formation in certain cells.

In summary, the present study demonstrated that GlcNAcβ1→6Manα1→R side chain itself is not a preferential site for poly-N-acetyllactosamine formation. Rather, addition of this side chain on GlcNAcβ1→2Manα1→R side chain by GnTv, forming GlcNAcβ1→6(GlcNAcβ1→2)Manα1→Manβ→R, converts the acceptor extremely favorable for poly-N-acetyllactosamine formation (Fig. 4). Moreover, we found that the
branched acceptor formed is first galactosylated at GlcNAcβ-1→6Man side, which is a key step to add N-acetyllactosamine extension equally in both GlcNAcβ-1→6Man→R and GlcNAcβ-1→2Man→R side chains (Fig. 10). The present study, however, did not address why poly-N-acetyllactosamine are added more readily on membrane proteins than secretory proteins (57). Because the iGnT has a unique transmembrane domain (32), further studies will be of significance to address this point in relation to the actions of membrane-bound iGnT.

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