Regulation of I-Branch Poly-N-Acetyllactosamine Synthesis

CONCERTED ACTIONS BY i-EXTENSION ENZYME, I-BRANCHING ENZYME, AND β1,4-GALACTOSYLTRANSFERASE

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I-branched poly-N-acetyllactosamine is a unique carbohydrate composed of N-acetyllactosamine branches attached to linear poly-N-acetyllactosamine, which is synthesized by I-branching β1,6-N-acetylglucosaminyltransferase. I-branched poly-N-acetyllactosamine can carry bivalent functional oligosaccharides such as sialyl Lewisx, which provide much better carbohydrate ligands than monovalent functional oligosaccharides. In the present study, we first demonstrate that I-branching β1,6-N-acetylglucosaminyltransferase cloned from human PA-1 embryonic carcinoma cells transfers β1,6-linked GlcNAc preferentially to galactosyl residues of N-acetyllactosamine close to nonreducing terminals. We then demonstrate that among various β1,4-galactosyltransferases (β4Gal-Ts), β4Gal-TI is most efficient in adding a galactose to linear and branched poly-N-acetyllactosamines. When a β1,6-GlcNAc branched poly-N-acetyllactosamine was incubated with a mixture of β4Gal-TI and i-extension β1,3-N-acetylglucosaminyltransferase, the major product was the oligosaccharide with one N-acetyllactosamine extension on the linear Galβ1→4GlcNAcβ1→3 side chain. Only a minor product contained galactosylated I-branch without N-acetyllactosamine extension. This finding was explained by the fact that β4Gal-TI adds a galactose poorly to β1,6-GlcNAc attached to linear poly-N-acetyllactosamines, while β1,3-N-acetylglucosaminyltransferase and β4Gal-TI efficiently add N-acetyllactosamine to linear poly-N-acetyllactosamines. Together, these results strongly suggest that galactosylation of I-branch is a rate-limiting step in I-branched poly-N-acetyllactosamine synthesis, allowing poly-N-acetyllactosamine extension mostly along the linear poly-N-acetyllactosamine side chain. These findings are entirely consistent with previous findings that poly-N-acetyllactosamines in human erythrocytes, PA-1 embryonic carcinoma cells, and rabbit erythrocytes contain multiple, short I-branches.

Poly-N-acetyllactosamines are unique glycans having N-acetyllactosamine repeats, (Galβ1→4GlcNAcβ1→3)n (1). Poly-N-acetyllactosamines are attached to N-glycans (2–5), O-glycans (6, 7), and glycolipids (8–10) and can be digested by endo-β-galactosidase (11). Poly-N-acetyllactosamines are often modified to express differentiater antigens and functional oligosaccharides. Among those oligosaccharides, sialyl Lewisx (Le*) and its sulfated forms are ligands for E-, P-, and L-selectin (12–16). During inflammation, E- and P-selectin expressed on activated endothelial cells bind to sialyl Le* oligosaccharides present on granulocytes and such initial binding leads to the extravasation of granulocytes. L-selectin on lymphocytes, on the other hand, recognizes sulfated sialyl Le* expressed in high endothelial venules of blood vessels (15, 16). This L-selectin/counterreceptor interaction allows lymphocytes to migrate into lymphoid system, allowing lymphocytes to circulate fully in the body.

In human granulocytes, monocytes, and certain T lymphocytes, poly-N-acetyllactosamines contain Le*, Galβ1→4(Fucα1→3)GlcNAc→R, and sialyl Le*, NeuNAcβ2→3Galβ1→4(Fucα1→3)GlcNAc→R (17, 18). In contrast, poly-N-acetyllactosamines in human erythrocytes contain ABO blood group antigens, synthesized from the precursor structure, Fucα1→2Galβ1→4GlcNAc→R (5, 19, 20). In addition, poly-N-acetyllactosamines can contain I-branches, Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→6Gal→R. During development of human erythrocytes, the linear i antigen represented by Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc→R is converted to those containing I-branches (21). In early mouse embryonic development, embryos express I antigen, which is gradually replaced with i antigen during development (2, 22).

The acquisition of I-branches is important, since two of the N-acetyllactosamine side chains can have functional terminal structures. It has been demonstrated that multivalent sialyl Le* poly-N-acetyllactosamines inhibit i-selectin-mediated binding and the rejection of organ transplants with much better efficacy than monovalent sialyl Le* poly-N-acetyllactosamines (23, 24). Similarly, blood group H antigens present at both termini in branched poly-N-acetyllactosamines were shown to have much better avidity to anti-ABO antibodies than linear poly-N-acetyllactosamines containing single antigenic structures (25). It was suggested that expression of i antigen in fetal erythrocytes minimizes a detrimental immune response when mother and fetus have incompatible blood group antigens (25).

These results, as a whole, indicate the significance of understanding how linear and I-branched poly-N-acetyllactosamines are synthesized. To this end, we have cloned cDNAs encoding β1,3-N-acetylglucosaminyltransferase (iGnT) that forms linear
poly-N-acetyllactosaminyltransferase (IGNt) that forms I-branches (27). The IGNt cloned was found to add β1,6-N-acetyllactosamine at the central galactose (underlined) of Galβ1-4GlcNAcb1-3Galβ1-4GlcNAc-R, thus termed as centrally acting IGNt (cIGNt) (28, 29). In addition, another IGNt, distally acting IGNt (dIGNt), was found to add β1,6-N-acetyllactosamine to peridistal galactose (underlined) of GlcNAcb1-3Galβ1-4GlcNAc-R (29–33). No studies have been reported, however, to determine how I-branched poly-N-acetyllactosaminyltransferase products are synthesized by IGNt, IGNt, and β4Gal-T.

In the present study, we first describe how the IGNt cloned from PA-1 cells (27) adds I-branches to linear poly-N-acetyllactosaminyltransferase containing multiple acceptor sites. We then demonstrate that β4Gal-T is responsible for galactosylation in the synthesis of both linear and branched poly-N-acetyllactosaminyltransferase. Finally, we reconstituted the synthesis of I-branched poly-N-acetyllactosaminyltransferase, the structure of which resembles that present in human erythrocytes (5), PA-1 human embryonic carcinoma cells (34), and rabbit erythrocytes (10). The results demonstrate an intricate interaction between acceptor substrates and these glycosyltransferases.

EXPERIMENTAL PROCEDURES

Isolation of cDNA Encoding IGNt and IGNt—cDNA encoding IGNt was cloned into pcDNA3.1, resulting in pcDNA1-IGNt, as described previously (26). pcDNA1-A, 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 constructed into this vector, resulting in pcDNA1-AIGNt.

cDNA encoding IGNt was cloned from a cDNA library constructed from human PA-1 embryonic carcinoma cells, resulting in pcDNA1-IGNt, as described previously (27). A catalytic domain of IGNt was prepared by polymerase chain reaction using pcDNA1-IGNt as a template. 5'- and 3'-primers for this polymerase chain reaction were 5'-CGCCAGATCCAGCTGGGT-3' and 5'-GGCTCGAGCTCATAAAATTACCGCTGGT-3' (BamHI and XhoI sites are underlined). The polymerase chain reaction product encoding amino acid residues 30–400 of the IGNt was digested with XhoI and then cloned into the same sites of pcDNA1-A, resulting in pcDNA1-AIGNt.

Expression of the Protein A-IGNt and Protein A-IGNt Fusion Protein—pcDNA-A, pcDNA1-AIGNt, and pcDNA1-AIGNt were separately transfected with Lipofectamine Plus (Life Technologies) into COS-1 cells as described previously (36). The chimeric enzyme released into serum-free Opti-MEM was used after adsorbing the Protein A-IGNt fusion protein to IgG-Sepharose 6FF (Amersham Pharmacia Biotech) as described previously (37). Alternatively, the culture medium was harvested by a Centricron 10 filter (Amicon), and GlcNAc was directly used as an enzyme source. In most of the studies, the concentrated culture medium was used for IGNt, since IgG-Sepharose-bound enzymes had a low activity as seen for other glycosyltransferases (38, 39). Typically, the activities of IGNt and IGNt in the incubation mixture were 38.0 nmol/h/ml using 0.5 mM Galβ1-4Glcβ-p-nitrophenol (Toronto Research Chemicals) and 40.0 nmol/h/ml using 0.5 mM Galβ1-4GlcNAcb1-3Galβ1-4GlcNAc-R6Manα1-6Manβ8-OC(1H)0,2CH2(1octyl), where n = 0, 1, and 2, were synthesized, starting from the derivatives of Galβ1-4GlcNAc-R and Manα1-6Manβ8-octyl, as described previously (36). GlcNAcβ1-3Galβ1-4GlcNAcβ1-6Manα1-6Manβ8-octyl and GlcNAcb1-3Galβ1-4GlcNAc-R6Manα1-6Manβ8-octyl were prepared by Escherichia coli β-galactosidase treatment of Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-R6Manα1-6Manβ8-octyl, and galactosyltransferase, as described previously (31). Purification of the product was achieved by HPLC on a TSK gel ODS-80Ts column (4.6 × 250 mm; Tosoh) equipped with a Gilson 306 HPLC apparatus. The column was equilibrated with 20 mM ammonium acetate buffer, pH 4.0, and eluted with the same buffer at a flow rate of 1.0 ml/min. Fluorescence was detected with a fluorescent spectrophotometer (Shimadzu, model RF-535) with excitation and emission wavelengths of 320 and 400 nm, respectively. The concentration of PA-oligosaccharides was estimated by comparing fluorescent intensity of synthesized PA-oligosaccharides and standard PA-glucose purchased from Takara Shuzo (PanVera, Madison, WI). Lacto-N-neo-tetraose, Lacto-N-neo-hexaose, and Lacto-N-neo-tetraose (PA-lacto-N-neo-tetraose) were used. The usages derived from the above oligosaccharides were purified by QAE-Sepharose A-25 gel and subjected to Bio-Gel P-4 gel filtration as described previously (36). The radioactivity of the aliquots was determined by a scintillation counter. The products derived from the PA-oligosaccharides were filtered through Ultrafree-MC (10-kDa cut; Millipore Corp.) and applied to the same ODS-80Ts column and eluted as described above. Since PA-oligosaccharides can be detected by fluorescence, non-radioactive UDP-GlcNAc was used as a donor for the experiments using PA-oligosaccharides.

To assay the transfer of N-acetyllactosamine residues by IGNt, the reaction mixture was exactly the same as described previously (36). As acceptors, lacto-N-neo-tetraose, lacto-N-neo-hexaose, lacto-N-hexaose, pyridylnamidated lacto-N-neo-hexaose (PA-lacto-N-neo-hexaose), and lacto-N-neo-tetraose (PA-lacto-N-neo-tetraose) were used. The usages derived from the above oligosaccharides were purified by QAR-Sephadex A-25 gel and subjected to Bio-Gel P-4 gel filtration as described previously (36). The radioactivity of the aliquots was determined by a scintillation counter. The products derived from the PA-oligosaccharides were filtered through Ultrafree-MC (10-kDa cut; Millipore Corp.) and applied to the same ODS-80Ts column and eluted as described above. Since PA-oligosaccharides can be detected by fluorescence, non-radioactive UDP-GlcNAc was used as a donor for the experiments using PA-oligosaccharides.

Substrate Specificity of β4Gal-T—β4Gal-T was prepared as described above. The product was purified by HPLC using NH3-bonded silica column (Varian Micropak AX-5) as described previously (36). The radioactivity of the aliquots was determined. 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.

Substrate Specificity of β4Gal-T, TT, TH, TV, and TV—Assays of β4Gal-Ts were performed exactly as described previously (36). As acceptors, GlcNAcβ1-3Galβ1-4Glcβ-p-nitrophenol—PA and Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-p-nitrophenol—PA were used. Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-p-nitrophenol—PA was synthesized by incubating 5 mM Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-p-nitrophenol—PA with the recombinant IGNt and 5 mM UDP-GlcNAc for 10 h in 100 μl of the reaction mixture as described above. The product was purified by HPLC using the same ODS column as described above and used as an acceptor.

Poly-N-acetyllactosamine Formation in I-Branches Oligosaccharide—To assay poly-N-acetyllactosamine formation, 0.5 mM Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-p-nitrophenol—PA with β4Gal-T (152 nmol/h/ml), IGNt (38 nmol/h/ml), 0.5 mM UDP-GlcNAc, and 0.5 mM UDP-Gal in 50 μl of 100 mM cacodylate buffer, pH 7.0, containing 20 mM MnCl2 and 10 mM each of GlcNAc-1,5-lactone and Gal-1,5-lactone. After incubation at 37 °C for 4 h, the reaction mixture was filtered and subjected to HPLC as described above. In these experiments, the incubation condition was first determined using Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-p-nitrophenol, where only one N-acetyllactosamine
unit can be added. In addition, β4Gal-TI was 4-fold in excess over iGnT, the same ratio as present in HL-60 cells (44).

**Analysis of Products by Endo-β-Galactosidase Digestion—** Products were digested with *Escherichia freundii* endo-β-galactosidase for 18 h at 37 °C (11). The digestion condition used allowed the cleavage of galactose linkage, where no β1,6-linked N-acetylgalactosamine is attached (11, 45). The digests were subjected to HPLC using AX-5 column or TSK gel ODS-80TS.

**RESULTS**

**Addition of I-branch to Various Poly-N-Acetyllactosaminyl Side Chains**—Recently, it was reported that the IGnT cloned from PA-1 cells exclusively adds β1,6-linked N-acetylgalactosamine to a galactose residue in a central position as seen in Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc (Fig. 2, compound c). These results indicate that the IGnT displays a preference for the recombinant IGnT bound to IgG-Sepharose, the activity of which was 3.6 nmol/h/ml. Almost identical results were obtained when a concentrated culture medium from IGnT-transfected cells, of which activity was 40.0 nmol/h/ml, was used.

**β4Gal-TI Is Responsible for Galactosylation of Linear and Branched Poly-N-acetyllactosaminyl Synthesis**—In our previous study, we demonstrated that β4Gal-TI is involved in N-acetyllactosaminyl formation in N-glycans, while β4Gal-TIV forms N-acetyllactosaminyl in core 2 branched O-glycans (36).

**Addition of Multiple, Short I-Branches**

The above results were obtained using the recombinant IGnT bound to IgG-Sepharose, the activity of which was 3.6 nmol/h/ml. Almost identical results were obtained when a concentrated culture medium from IGnT-transfected cells, of which activity was 40.0 nmol/h/ml, was used.

**β4Gal-TI** transfers a galactose most efficiently to this acceptor, whereas β4Gal-TII, -TIII, and -TV exhibit substrate inhibition at higher concentrations of the acceptor substrate, probably because β-galactose in the acceptor competes with UDP-Gal (Fig. 3A). Although β4Gal-TIV does not exhibit substrate inhibition, it is less efficient than β4Gal-TI (Table 1).

It is also evident that β4Gal-TI, among these β4Gal-Ts, acts...
most efficiently on a linear poly-N-acetyllactosamine acceptor, GlcNAcβ1–3Galβ1–4Glc–PA (Fig. 3B, Table I). In both experiments, similar results were obtained when the concentration of these enzymes was increased 5-fold or decreased 5-fold. These results indicate that β4Gal-TI is mostly responsible for galactosylation of both branched and linear poly-N-acetyllactosamines.

Addition of Multiple, Short I-Branches

Addition of N-Acetylgalactosamine to I-branched Acceptors—To determine how iGnT adds N-acetylgalactosamine residues to I-branched oligosaccharides, the incorporation of N-acetylgalactosamine was compared between different acceptors including linear and branched oligosaccharides. The results shown in Fig. 4 demonstrate that lacto-N-neo-hexaose did not incorporate twice the amount of N-acetylgalactosamine compared with lacto-N-neo-tetraose, despite the fact that the former contains two acceptor sites (A and B). Moreover, lacto-N-hexaose containing only one acceptor galactose in the I-branch incorporated N-acetylgalactosamine as much as lacto-N-neo-hexaose (Fig. 4C). The results strongly suggest that the addi-
Addition of Multiple, Short I-Branches

FIG. 4. IγnT activity on linear and branched N-acetyllactosaminyl oligosaccharides. Galβ1→4GlcNAcβ1→3Galβ1→4Glc (A), Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Glc (B), and Galβ1→3GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Glc (C) were incubated with the IγnT and UDP-[3H]GlcNAc for 10 h. The products were analyzed by Bio-Gel P-4 gel filtration, and numbers shown in the upper right indicate the relative amount of incorporated radioactivity. A peak eluted at fraction 51 was due to a contaminant derived from UDP-[3H]GlcNAc.

Addition of N-acetylgallosaccharide to one side chain precludes the addition of another GlcNAc to the other side chain in branched structures.

To determine the structures of products, the enzymatic reaction products derived from PA-lacto-N-neo-hexaose were subjected to HPLC using an ODS column. Two products, peak A and B, were then separately digested with endo-β-galactosidase followed by exo-β-N-acetylgalactosaminidase treatment (Fig. 5). The results indicate that peak A, the major product (75% of peak B contains substrate. This structure was further confirmed by endo-β-N-acetylgalactosaminidase digestion followed by the addition of one galactose to the I-branch in the acceptor Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Glc→PA (Fig. 7).

The minor peak of the products, peak F, eluted shortly after the major peak and corresponds to Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Glc→PA, which represents the addition of one galactose to the branched acceptor substrate. This structure was further confirmed by endo-β-galactosidase digestion and exoglycosidase digestion (data not shown). The major peak (84% of the total products), peak E, was digested by endo-β-galactosidase, followed by β-N-acetylgalactosaminidase (Fig. 7, C and D). This digested material was eluted at the position corresponding to Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Glc→PA (Fig. 7D), which was also obtained in the above experiment (Fig. 5H). These results indicate that one potential acceptor site is missed, precluding the addition of another branch on the same side chain (compound E in Fig. 8) or lacto-N-neo-hexaose (compound F in Fig. 8).

Since the majority of the product was compound E, the extension of N-acetyllactosamine units along the linear poly-N-acetyllactosaminyl side chain is favored over galactosylation of the branched acceptor side chain (compound F in Fig. 8). These results are entirely consistent with the previous findings that I-branches are usually composed of only one N-acetyllactosamine unit in erythrocytes (5) and human PA-1 embryonic carcinoma cells (34) from which the eGnT was cloned.

Galactosylation of β1,6-GlcNAc Branch Is a Rate-limiting Step—The above results demonstrate that I-branch formation was much lower affinity toward the branched acceptor Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Glc→PA (K_m = 2.49 mM) than its linear counterpart GlcNAcβ1→3Galβ1→4Glc→PA (K_m = 0.31 mM). In contrast, iGnT exhibits higher affinity toward the branched acceptor Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Glc→PA (K_m = 0.52 mM) than the linear acceptor Galβ1→4GlcNAcβ1→3Galβ1→4Glc→PA (K_m = 1.09 mM) (Figs. 3, C and D, Table II).

These results indicate that β1,3-linked GlcNAc is added to the Galβ1→4GlcNAcβ1→3Gal side chain before galactosylation of β1,6-linked GlcNAc branch (Fig. 8C). This reaction is more likely, immediately followed by galactosylation of the GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Gal moiety (Fig. 8D), considering that the additions of GlcNAc and Gal to Galβ1→4GlcNAcβ1→3Gal are favored by these two enzymes (Tables I and II). As the last step, galactosylation of I-branch takes place (Fig. 8E). Only as a minor biosynthetic pathway, galactosylation of I-branch precedes other reactions forming compound F in Fig. 8, which may lead to more complex poly-N-acetyllactosaminyls.

DISCUSSION

The present study demonstrated that I-branch formation takes place more efficiently at sites closer to nonreducing termini than at internal sites, suggesting that I-branch is formed preferentially at the end of elongating poly-N-acetyllactosaminyl side chain. To understand how this is achieved, Km and V_max values of β4Gal-TI and iGnT were obtained for linear and branched oligosaccharide acceptors. As shown in Table I, β4Gal-TI exhibits much lower affinity toward the branched acceptor Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Glc→PA (K_m = 2.49 mM) than its linear counterpart GlcNAcβ1→3Galβ1→4Glc→PA (K_m = 0.31 mM). In contrast, iGnT exhibits higher affinity toward the branched acceptor Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Glc→PA (K_m = 0.52 mM) than the linear acceptor Galβ1→4GlcNAcβ1→3Galβ1→4Glc→PA (K_m = 1.09 mM) (Figs. 3, C and D, Table II).

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located more than one $N$-acetyllactosaminyl unit apart. Similar results were obtained on the ceramide pentadecasaccharide from rabbit erythrocytes (10). This spacing may be necessary for other modifications, since I-branch and A-blood group terminal structure are separated by two $N$-acetyllactosamine units in A$^d$ glycolipid from human erythrocytes (8). The iGnT

**Fig. 5.** HPLC analysis of the products after incubation of PA-lacto-$N$-neo-hexaose with iGnT. The products obtained after incubation of Galβ1→4GlcNAcβ1→3(Galβ1→4GlcNAcβ1→6)-Galβ1→4Glc→PA with the iGnT and UDP-GlcNAc were applied to HPLC (A, E). Peak A (B–D) and peak B (F–H) were purified (B and F) and then sequentially digested with endo-β-galactosidase (C and G) and exo-β-$N$-acetylglucosaminidase (D and H) and analyzed by the same HPLC. Peak S corresponds to the starting material. ODS-80TS column was used in this HPLC.

**Fig. 6.** Structures of the iGnT products from PA-lacto-$N$-neo-hexaose. The products after incubation of PA-lacto-$N$-neo-hexaose with the iGnT are shown. The products, obtained after sequential treatment of endo-β-galactosidase and β-$N$-acetylglucosaminidase, are also shown. Peaks A and B correspond to A and B in Fig. 5, respectively.
was cloned from a cDNA library of human PA-1 embryonic carcinoma cells (27). These results suggest that human and rabbit erythroid precursor cells contain IgNt that is the same as or similar to the IgNt cloned from PA-1 cells. Hog small intestine may have similar poly-N-acetyllactosamine structures as seen in erythrocytes and PA-1 cells, since a cIgNt was purified from this tissue (46).

The present study also demonstrated that intrinsic properties of β4Gal-TI and iGnT are critical in forming short I-branches. The addition of a galactose residue to β1,6-linked N-acetylgalactosamine branch is a much slower process than the addition of N-acetylgalactosamine or galactose to an elongating Galβ1→4GlcNAcβ1→3 side chain, as demonstrated by kinetic data (Table I and II). The addition of β1,3-linked N-acetylgalactosamine to the Galβ1→4GlcNAcβ1→3 side chain is even more efficient when this acceptor has a β1,6-linked GlcNAc branch (Table II). Combined together, these results indicate that galactosylation of a GlcNAcβ1→6 branch takes place as a rate-limiting step in the synthesis of branched poly-N-acetyllactosamines. This is most likely the reason why no elongation of I-branch was observed in the present study when the acceptor containing β1,6-linked GlcNAc was incubated with a mixture of iGnT and β4Gal-TI (Figs. 7 and 8).

These results are entirely consistent with the structures determined on poly-N-acetyllactosamines from human erythrocyte band 3 (5), human PA-1 embryonic carcinoma cells (34), and rabbit erythrocytes (10). Only one N-acetyllactosamine unit is present in each I-branch of these glycans.

The results obtained in the present study predict that it takes longer for additional modifications of formed I-branch than the extension of Galβ1→4GlcNAcβ1→3 side chain. If this is the case, I-branches in internal positions contain fewer modifications than I-branches at nonreducing termini. Similarly, additional modification at elongating linear poly-N-acetyllactosamines should take place faster than at I-branches even at nonreducing termini. In fact, more α1,2-fucosylated I-branch was found in those branches at nonreducing termini than those in internal positions in human band 3 (5). Moreover, Fucα1→2Galβ1→4GlcNAcβ1→3(Galβ1→4GlcNAcβ1→6)Galβ1→R but not Galβ1→4GlcNAcβ1→3(Fucα1→2Galβ1→4GlcNAcβ1→6)Galβ1→R was found in monofucosylated termini of human band 3 (5). Thus, all of these structural data are consistent with the results predicted from the present study, demonstrating that galactosylation of I-branch is a rate-limiting step.

It is noteworthy that the iGnT can act very efficiently on Galβ1→4GlcNAcβ1→3(GlcNAcβ1→6)Galβ1→4Glc→PA (Fig. 3C and Table II) but not on Galβ1→4GlcNAcβ1→3(GlcNAcβ1→6)Galβ1→4Glc→PA (Fig. 5, peak A). This result suggests that short GlcNAcβ1→6 branch may not be recognized by the iGnT, while the terminal GlcNAc residue in the extended GlcNAcβ1→3Galβ1→4GlcNAcβ1→6 branch may be recognized by the iGnT, preventing the addition of GlcNAc to the other side chain due to a substrate inhibition. It is thus tempting to speculate that the short GlcNAcβ1→6 branch attached to linear poly-N-acetyllactosamines may be difficult for β4Gal-TI to recognize because of its conformation. In fact, NMR studies on the pentadecaglycolipid indicate that the anomeric proton of β1,6-GlcNAc linked to the internal galactose is not detected, suggesting that it is conformationally inaccessible (10). These results, combined together, indicate that intricate interaction between these glycosyltransferases and I-branched acceptors play a critical role in the synthesis of I-branched poly-N-acetyllactosamines.

Previously, it was determined that core 2 branch GlcNAcβ1→4Glcα1→6(Galβ1→3)GalNAcα→R is galactosylated most efficiently
FIG. 8. Proposed biosynthetic steps of I-branched poly-N-acetyllactosamine. β1,6-linked N-acetylgalactosamine is first added to a central galactosyl donor by α1,2 Gal-glctosyltransferase (GlcNAc, B). This is followed by the addition of β1,3-linked N-acetylgalactosamine by β1,3 Gal-glctosyltransferase (C) and β1,4-linked galactose by β1,4 Gal-glctosyltransferase (D), adding N-acetylgalactosamine to the linear poly-N-acetyllactosamine chain. This is followed by galactosylation of β1,6 linked N-acetyllactosamine, forming I-branch (E). As a minor biosynthetic pathway, galactosylation of I-branch may take place as soon as β1,6-linked N-acetyllactosamine is added (F). If compound F is formed, β1,3-linked N-acetyllactosamine is preferentially added to I-branch by β1,3 Gal-glctosyltransferase, potentially leading to more complex poly-N-acetyllactosamines. E and F correspond to peaks E and F in Fig. 7, respectively. This biosynthetic pathway is based on the results obtained in the present study.

### Table II

**Kinetic properties of iGnT**

| Substrate | $K_m$ (mM) | $V_{max}$ (relative values) |
|-----------|-----------|----------------------------|
| Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA | 1.09 | 100% |
| Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA | 0.52 | 150% |

* $V_{max}$ of iGnT is compared with the $V_{max}$ (55.4 mmol/h/ml) obtained using Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA as an acceptor.*

by β1,4 Gal-glctosyltransferase (36). Other galactosylderivatives such as β1,4 Gal-glctosyltransferase, -TII, -TIII, and -TV exhibit a substrate inhibition toward the core 2 acceptor, probably because of competition between β1,4 galactose in the acceptor and the donor substrate, UDP-Gal. Similarly, β1,4 Gal-glctosyltransferase, -TII, -TIII, and -TV exhibited a substrate inhibition toward the I-branched acceptor (Fig. 3A). β1,6 Gal-glctosyltransferase and β1,4 Gal-glctosyltransferase did not show a substrate inhibition toward the I-branch acceptor. However, the kinetic efficiency ($V_{max}/K_m$) for β1,4 Gal-glctosyltransferase is less than a half of that for β1,6 Gal-glctosyltransferase (Table I), indicating that β1,4 Gal-glctosyltransferase is probably dominant in I-branch formation. β1,6 Gal-glctosyltransferase is most efficient in galactosylation of a linear poly-N-acetyllactosamine as well (Fig. 3B and Table I). Moreover, β1,4 Gal-glctosyltransferase is most efficient in the synthesis of N-glycan poly-N-acetyllactosamine as shown in the previous study (36). Overall, these results indicate that β1,4 Gal-glctosyltransferase plays a major role in poly-N-acetyllactosamine extension and I-branch formation in N-glycans. It is noteworthy that β1,6 Gal-glctosyltransferase knock-out mice survive during development (47), and those mutant mice express polysialic acid (38) and the HNK-1 carbohydrate epitope (48) in brain glycoproteins (49). These results suggest that a β1,4 Gal-glctosyltransferase other than β1,6 Gal-glctosyltransferase partly compensates for the loss of β1,4 Gal-glctosyltransferase in the knock-out mice and is possibly involved in N-acetyllactosamine synthesis under normal conditions as well.

The experiments carried out in the present study were designed to mimic cellular biosynthetic pathways. The biosynthetic oligosaccharide products are also a result of the balance between the amount of glycoconjugates present and the movement of glycoconjugates in the Golgi apparatus during biosynthesis (50). For large scale synthesis of oligosaccharides in vitro, however, enzymatic synthesis can be achieved despite the fact that such a reaction is unlikely in vivo. For example, Renkonen et al. (51) synthesized highly branched poly-N-acetyllactosaminyl oligosaccharides containing four sialyl Leα termini using the cGnT. In this oligosaccharide, every possible acceptor site was occupied by I-branch and all of the I-branches contained sialyl Leα. It was also reported that galactosylation of core 2 branch GlcNAcβ1-6Galβ1-3GalNAc could be achieved using an excess amount of β1,6 Gal-glctosyltransferase (36, 52), although β1,6 Gal-glctosyltransferase is unlikely to be involved in its galactosylation in vivo. These results strongly suggest that the results obtained by in vitro studies need to be evaluated regarding how these findings reflect the biosynthesis taking place in cells.

The present study reveals the biosynthetic pathway involving the cGnT that adds β1,6-linked GlcNAc to central galactose residues. It has been demonstrated that there is an additional Ig receptor, dGnT, which adds β1,6-linked GlcNAc at peridistal galactose residues, forming GlcNAcβ1→3(GlcNAcβ1→6)Galβ1→R at nonreducing termini (30–33). In this situation, galactosylation at I-branch may not be a rate-limiting step, since no substrate inhibition takes place. Recently, we have cloned a novel β1,6-N-acetylgalactosaminyltransferase that has more dGnT activity than cGnT activity (29). Future studies will be of significance to determine the structures of I-branched poly-N-acetyllactosamines synthesized by this newly cloned enzyme.

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