Enzymatic Midchain Branching of Polylactosamine Backbones Is Restricted in a Site-Specific Manner in α1,3-Fucosylated Chains†

Anne Leppänen, Ritva Niemelä, and Ossi Renkonen*

Institute of Biotechnology and Department of Biosciences (Division of Biochemistry), University of Helsinki, Biocenter 1, P.O. Box 56, FIN-00014 Helsinki, Finland

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ABSTRACT: Branched polylactosamines on animal cell surfaces are believed to contribute to multivalent interactions in cell adhesion and cell signalling. Their biosynthesis proceeds *via* linear precursors that become branched by β1,6-GlcNAc transferases (IGnT6, GlcNAc to Gal). Previous work has identified the tetrasaccharide Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc (**1**) and the hexasaccharide Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc (**4**) as acceptors for a rat serum enzyme activity (cIGnT6), which transfers GlcNAcβ1−6 units to the midchain galactose residues. Thereby, **1** is converted to the branched pentasaccharide Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAc and **4** to the doubly branched octasaccharide Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAc [Leppänen, A., Salminen, H., Zhu, Y., Maaheimo, H., Helin, J., Costello, C. E., & Renkonen, O. (1997) *Biochemistry 36*, 7026−7036]. Here we report that neither the α1,3-fucose-containing derivatives of **1** [Galβ1−4GlcNAcβ1−3Galβ1−4(Fucα1−3)GlcNAc and Galβ1−4(Fucα1−3)GlcNAcβ1−3Galβ1−4GlcNAc] nor a similar derivative of **4** [Galβ1−4GlcNAcβ1−3Galβ1−4(Fucα1−3)GlcNAcβ1−3Galβ1−4GlcNAc] were acceptors for the rat serum cIGnT6 activity. Hence, the enzyme's branch-forming action was completely prevented at sites in the immediate neighborhood of the fucosylated *loci* of the polylactosamines. In Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4(Fucα1−3)GlcNAc, the inhibition of the branch-forming reaction was restricted to the fucose-carrying LacNAc unit; at the middle LacNAc, the branching proceeded normally. However, in the isomeric Galβ1−4(Fucα1−3)GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc, the fucose residue prevented branching completely at the middle LacNAc and almost completely at the reducing end LacNAc. In summary, α1,3-fucose residues in polylactosamine chains inhibited the cIGnT6 reaction in a site-specific manner, at the fucosylated LacNAc unit itself and also at sites one and two LacNAc units upstream, but not at the LacNAc units downstream from the fucosylated *locus*. These data imply that site-directed branching in polylactosamines is possible *in vitro* with the aid of specifically positioned α1,3-fucosyl units, that can be removed afterward without harming the branched backbones.

Branched polylactosamines capped by multiple protein-binding sugar sequences possess potentially valuable anti-inflammatory as well as contraceptive properties. Multiply branched polylactosaminoglycans carrying several sialyl Lewis x (sLex)[1] epitopes have proven themselves as L-selectin antagonists of very high potency *in vitro* (Maaheimo et al., 1995; Turunen et al., 1995; Seppo et al., 1996; Renkonen et al., 1997; Toppila et al., 1997). Branched polylactosamine backbones are also very good scaffolds for presenting multiple adhesion-inhibiting Galα1−3Galβ1−4GlcNAc determinants to sp56, the sugar-binding sperm protein that mediates mouse gamete adhesion (Litscher et al., 1995; Niemelä et al., 1995b; Seppo et al., 1995). Analogous data, emphasizing the importance of multivalency of ligands, are available concerning other interactions between carbohydrates and lectins on cell and virus surfaces.

At present, the critical structural features of efficient polylactosamine antagonists for the selectins and the sp56 protein are poorly understood. Structure *versus* function studies would benefit greatly if different oligosaccharides, structurally related to the known antagonists could be systematically synthesized. For instance, methods that would allow site-specific positioning of protein-binding sugar epitopes in multiple copies along polylactosamine backbones would be highly desirable. The solution of this problem requires site-specific branching of linear polylactosamines.

Linear polylactosamine backbones are branched by two distinct types of β1,6-GlcNAc transferase (IGnT6) activities, both of which are able to transform linear acceptors to branched blood group I type sequences in conjunction with the nearly ubiquitous β1,4-galactosyltransferase. The "distally acting" IGnT6 (dIGnT6) activities transfer to the subterminal galactose of a growing polylactosamine chain,

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* To whom correspondence should be addressed. Tel: 358-9-708 59375. Fax: 358-9-708 59563. E-mail: ossi.renkonen@helsinki.fi.

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[1] Abbreviations: cIGnT6, centrally acting β1,6-*N*-acetylglucosaminyltransferase (GlcNAc to Gal); dIGnT6, distally acting β1,6-*N*-acetylglucosaminyltransferase (GlcNAc to Gal); Fuc, L-fucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; LacNAc, *N*-acetyllactosamine (Galβ1−4GlcNAc); Lex, Lewis x, [Galβ1−4(Fucα1−3)-GlcNAc]; MALDI-TOF, matrix assisted laser desorption-ionization mass spectrometry with time-of-flight detection; MH, maltoheptaose [Glcα1−4(Glcα1−4)$_5$Glc]; MP, maltopentaose [Glcα1−4(Glcα1−4)$_3$Glc]; MT, maltotriose (Glcα1−4Glcα1−4Glc); MTet, maltotetraose [Glcα1−4(Glcα1−4)$_2$Glc]; NMR, nuclear magnetic resonance; sLex, sialyl Lewis x, [NeuNAcα2−3Galβ1−4(Fucα1−3)GlcNAc]; WGA, wheat germ agglutinin.

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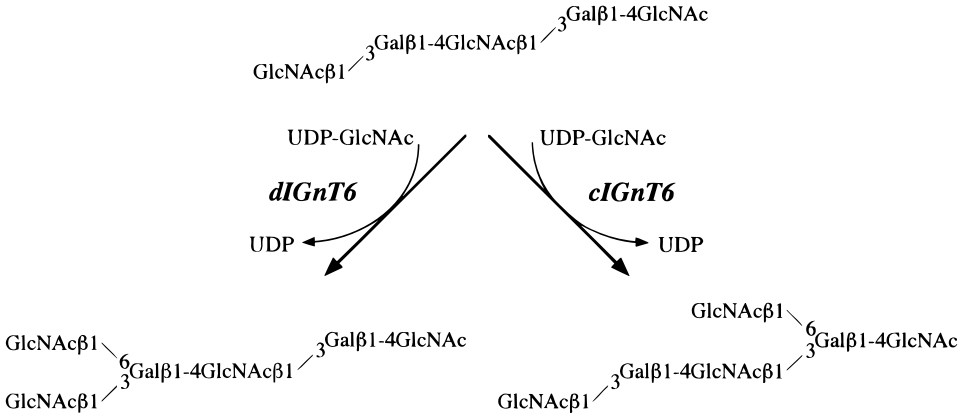
Scheme 1: The Distinction between the Distally Acting β1,6-*N*-Acetylglucosaminyltransferase (dIGnT6) and the Centrally Acting β1,6-*N*-Acetylglucosaminyltransferase (cIGnT6) Activities Is Highlighted by Generation of Branches at Different Sites in a Linear Pentasaccharide Acceptor (Leppänen et al., 1991; Helin et al., 1997)



transforming the trisaccharide GlcNAcβ1−3′LacNAc into the tetrasaccharide GlcNAcβ1−3′(GlcNAcβ1−6′)LacNAc (Piller et al., 1984; Brockhausen et al., 1986; Koenderman et al., 1987; Ropp et al., 1991; Gu et al., 1992) and the pentasaccharide GlcNAcβ1−3′LacNAcβ1−3′LacNAc into the hexasaccharide GlcNAcβ1−3′(GlcNAcβ1−6′)LacNAcβ1−3′LacNAc (Helin et al., 1997). The "centrally acting" IGnT6 (cIGnT6) activities, in turn, act on midchain galactose units of the acceptor, e.g., transforming the tetrasaccharide Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc/Glc into the branched pentasaccharide Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAc/Glc (Gu et al., 1992; Niemelä et al., 1995c; Maaheimo et al., 1997) and the pentasaccharide GlcNAcβ1−3′LacNAcβ1−3′LacNAc into the branched hexasaccharide GlcNAcβ1−3′LacNAcβ1−3′(GlcNAcβ1−6′)LacNAc (Leppänen et al., 1991, 1997). The latter is a positional isomer of the branched glycan generated by the distally acting enzyme as shown in Scheme 1.

The present report deals with deactivation of certain branching sites in long, fucose-containing polylactosamines. In transferring a β1,6-GlcNAc branch to the polylactosamine R_1-[Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAcβ1]-OR_2 at the underlined galactose residue, the cIGnT6 activity present in rat serum appears to bind to the bracketed tetrasaccharide sequence of the acceptor (Leppänen et al., 1997). Hence, we reasoned that prior α1,3-fucosylation of the "upstream" GlcNAc residue adjacent to the underlined galactose could inhibit the branch-forming cIGnT6 reaction. The assumption was based on the NMR-data showing that in solution the galactose and fucose rings of the trisaccharide Galβ1−4(Fucα1−3)GlcNAc are stacked to each other (Wormald et al., 1991; Ball et al., 1992; Ichikawa et al., 1992; Miller et al., 1992), masking several elements of the LacNAc unit. Accordingly, we initiated a program, aiming to enzyme-assisted synthesis of linear polylactosamine backbones that carry α1,3-fucosyl residues at predetermined sites. Having eventually succeeded in obtaining suitable fucosylated polylactosamines (Niemelä et al., 1995c; R. Niemelä, et al., unpublished experiments), we now report on their use in the branch-forming experiments with cIGnT6 activity present in mammalian blood serum. Indeed, the fucosylated polylactosamines proved to be site-specifically deactivated for the branching reactions as expected. Some of the present data have been reported in a preliminary form (Leppänen et al., 1994).

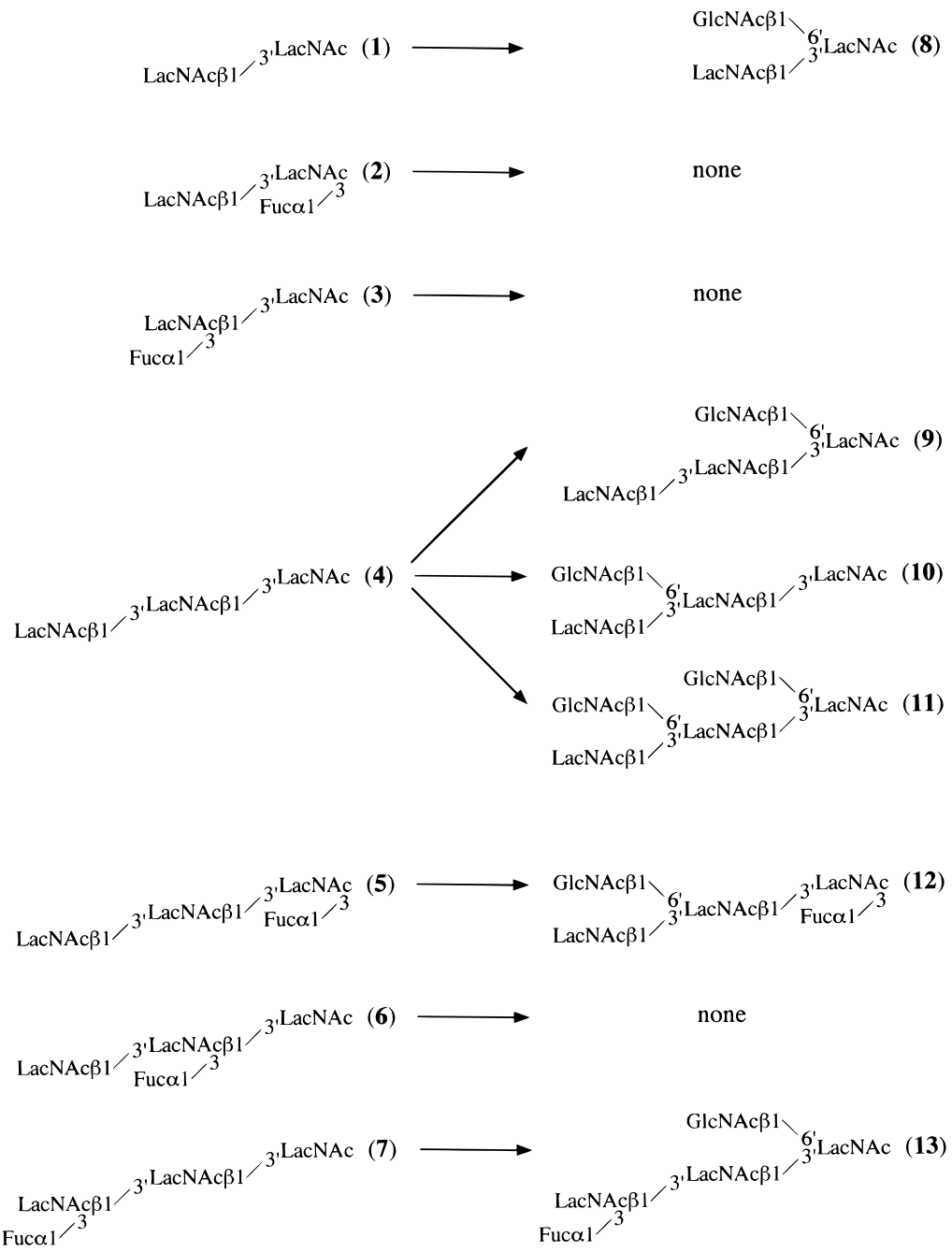
EXPERIMENTAL PROCEDURES

Oligosaccharides. The acceptor oligosaccharides of Table 1 were synthesized enzymatically as described: **1** (Renkonen et al., 1991b), **3** (Niemelä et al., 1995a), **4** (Leppänen et al., 1997), and **2**, **5**, **6**, and **7**, (R. Niemelä, et al., unpublished experiments). Briefly, glycan **2** was obtained from the tetrasaccharide Galβ1−4GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAc (Renkonen et al., 1991b) by a process involving (i) partial α1,3-fucosylation catalyzed by α1,3/4-fucosyltransferase(s) of human milk, (ii) chromatographic isolation of the resulting pentasaccharide fraction which contains two isomeric monofucosyl products, and (iii) isolation of glycan **2** from this mixture by chromatography on immobilized wheat germ agglutinin (WGA). Glycans **5** and **6** were obtained from the pentasaccharide GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc by a process involving (i) partial α1,3-[^{14}C]fucosylation at either of the two LacNAc units, (ii) chromatographic isolation of the resulting monofucosylated products in form of a hexasaccharide fraction, (iii) chromatographic fractionation of this mixture on immobilized WGA, and (iv) enzymatic β1,4-galactosylation of both pure hexasaccharides. Glycan **7** was obtained from [^{14}C]Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc by a process consisting of (i) partial α1,3-fucosylation, (ii) isolation of the heptasaccharide fraction, (iii) cleavage of glycans **5** and **6** by β-galactosidase and (iv) chromatographic isolation of intact glycan **7**. The structures of glycans **2**, **5**, **6**, and **7** were established by degradative processes, as well as by ^{1}H-NMR analysis and by MALDI-TOF mass spectrometry.

Glycan **8** was synthesized as described in (Niemelä et al., 1995c, Maaheimo et al., 1997). Marker oligosaccharides **9**, **10**, and **11** were synthesized as described in (Leppänen et al., 1997). The tetrasaccharide Galβ1−4([^{14}C]Fucα1−3)-GlcNAcβ1−3Gal was obtained as in (Renkonen et al., 1991a).

β1,6-GlcNAc Transferase Reactions. β1,6-GlcNAc transferase reactions with the midchain enzyme activity (cIGnT6) present in rat and human serum were performed essentially as described for human serum (Leppänen et al., 1991) and for rat serum (Leppänen et al., 1997). In short, 1−2 pmol of the radiolabeled acceptor saccharides were incubated with 145 mM UDP-GlcNAc, 50 mM Tris-HCl, pH 7.5, 8 mM NaN_3, 20 mM EDTA, 0.5 mM ATP, and 50 μL of

Table 1: Structures of Acceptor Saccharides and Products Obtained in Reactions Catalyzed by the cIGnT6 Activity of Rat Serum



unconcentrated rat serum or 50 μL of human serum concentrated by centrifugal ultrafiltration (the macromolecules of M_r > 10 kDa being concentrated about 2−3-fold). In addition, 60 mM D-galactonolactone and 200 mM D-galactose were added to inhibit endogenous β-galactosidase activity in reactions with rat serum. All pairs of fucose-containing and fucose-free acceptors of identical backbones were compared in parallel experiments under strictly identical conditions using equimolar amounts of the acceptors.

All reaction mixtures were desalted by passing them in water through a mixed bed of ion exchangers AG 50 $[H]^+$ and AG 1 $[OAc]^-$ (Bio-Rad, Richmond, CA) (Renkonen et al., 1989). The products were then separated from the acceptors by paper chromatography.

Glycosidase Reactions. Digestions with jack bean β-galactosidase (EC 3.2.1.23) (Sigma, St. Louis, MO) and *endo*-β-galactosidase from *B. fragilis* (EC 3.2.1.103) (Boehringer Mannheim, Germany) were carried out as in Renkonen et al. (1991b). All digests were desalted by using ion exchange resins.

Paper Chromatography. Paper chromatographic runs were performed on Whatman III Chr paper with the upper phase of 1-butanol/acetic acid/water (4:1:5, v/v; solvent A). Radiolabeled saccharides were detected as described (Renkonen et al., 1989), using Optiscint (Wallac, Turku, Finland) as scintillant. Marker lanes on both sides of each sample lane were stained with silver nitrate.

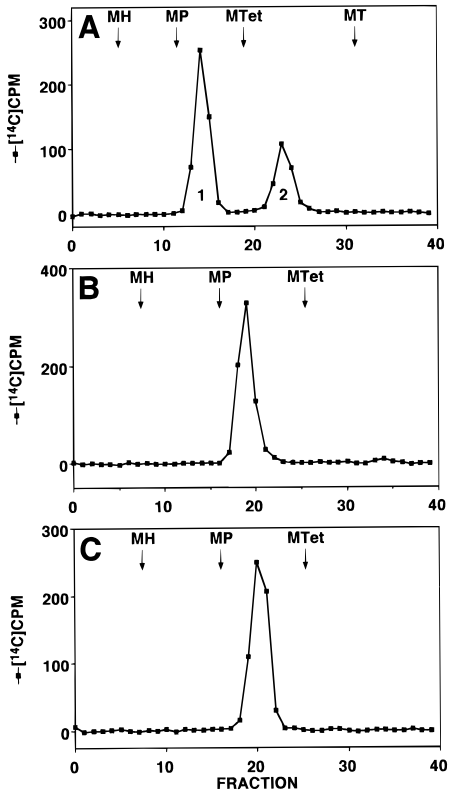


FIGURE 1: Paper chromatograms of oligosaccharides obtained when radiolabeled acceptor saccharides were incubated with UDP-GlcNAc and the cIGnT6 activity of rat serum. Arrows show the positions of malto-oligosaccharide markers, MT, maltotriose, MTet, maltotetraose, MP, maltopentaose, MH, maltoheptaose. (Panel A) Branch-formation in the fucose-free tetrasaccharide Galβ1−4GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAc (**1**). Solvent A, 133 h. Peak 1 represents the branched pentasaccharide product **8** and peak 2 is the tetrasaccharide acceptor **1**. (Panel B) Attempted branching of Galβ1−4GlcNAcβ1−3Galβ1−4([^{14}C]Fucα1−3)GlcNAc (**2**). Solvent A, 192 h. Only the unchanged acceptor is detected. (Panel C) Attempted branching of Galβ1−4([^{14}C]Fucα1−3)GlcNAcβ1−3Galβ1−4GlcNAc (**3**). Solvent A, 192 h. Only the unchanged acceptor is detected.

RESULTS

Central Backbone Branching of Polylactosamines Was Prevented at Galactose Units Adjacent to α*1,3-Fucosylated GlcNAc Residue.* An overview of the structures of the acceptors and products of the present experiments is shown in Table 1.

When a sample (2 pmol) of the tetrasaccharide Galβ1−4GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAc (**1**) was incubated with UDP-GlcNAc and the cIGnT6 activity present in rat serum, the branched pentasaccharide Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)[^{14}C]Galβ1−4GlcNAc (**8**) was formed in a yield of 66% (Figure 1A), confirming our previous data (Niemelä et al., 1995c; Maaheimo et al., 1997). However, in a parallel experiment, carried out with 2 pmol of the fucose-containing pentasaccharide Galβ1−4GlcNAcβ1−3Galβ1−4([^{14}C]Fucα1−3)GlcNAc (**2**), the reaction mixture contained only the original radiolabeled acceptor (R_{MTet} = 0.73, R_{MP} = 1.16) (Figure 1B). Slower moving products were not detected in chromatography, indicating that no β1,6-bonded GlcNAc residues were transferred to the acceptor. In another parallel experiment, performed with 2 pmol of the isomeric fucose-containing pentasaccharide Galβ1−4([^{14}C]Fucα1−3)GlcNAcβ1−3Galβ1−4GlcNAc (**3**), the reaction product again represented solely the unchanged radiolabeled acceptor (R_{MTet} = 0.80, R_{MP} = 1.27), with no slower moving products visible on the paper chromatogram (Figure 1C). Hence, the ability of the central galactose of glycan **1** to accept a β1,6-GlcNAc residue was abolished in glycans **2** and **3** where either the adjoining upstream GlcNAc or the adjoining downstream GlcNAc residue, respectively, was α1,3-fucosylated. Analogous data were obtained with the tetrasaccharide **1** and the fucose-containing pentasaccharides **2** and **3** when human serum was used as a source of the cIGnT6 activity (not shown).

When a 1.5 pmol sample of the hexasaccharide [^{3}H]-Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc (**4**) was subjected to a branch-forming experiment with UDP-GlcNAc and cIGnT6 activity present in rat serum, the reaction mixture revealed 38% of the doubly branched octasaccharide and 44% of a mixture of the singly branched heptasaccharides, together with 18% of the unreacted acceptor (Figure 2A), confirming our previous findings (Leppänen et al., 1997). The products cochromatographed with authentic markers of the octasaccharide **11** and the heptasaccharides **9** and **10**, respectively. *endo-*β-Galactosidase digestion of an analogous mixture of **9** and **10** has shown that 43% of the mixture consisted of **9** and 57% of **10** (Leppänen et al., 1997).

When the fucose-containing heptasaccharide Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4([^{14}C]Fucα1−3)GlcNAc (**5**) (1.5 pmol) was treated with UDP-GlcNAc and rat serum cIGnT6 activity in a parallel experiment with glycan **4**, an octasaccharide-like radiolabeled product was formed in a 55% yield (R_{MP} = 0.30; R_{MH} = 0.62); it chromatographed slower than the heptasaccharide acceptor (R_{MP} = 0.46; R_{MH} = 0.95) (Figure 2B), approximately at the same rate as the octasaccharide **11** in Figure 2A (peak 1). This suggests that only one β1,6-bonded GlcNAc was transferred to **5**, while two GlcNAc units were transferred to a significant fraction of the fucose-free glycan **4**. The product obtained from glycan **5** was completely resistant against the action of *endo-*β-galactosidase (not shown), while a complete cleavage of the heptasaccharide acceptor **5** at the central galactosidic bond was observed. The transfer-induced protection against *endo-*β-galactosidase established that the new β1,6-bonded GlcNAc unit had been transferred exclusively to the middle galactose of glycan **5**, generating glycan **12** (Scudder et al., 1984). We conclude that the α1,3-bonded fucose residue of the reducing end GlcNAc in heptasaccharide **5** prevented the cIGnT6 reaction at the reducing end LacNAc, as did the analogous fucose residue in pentasaccharide **2**. However, the fucose of glycan **5** did not block the branch formation at the middle LacNAc; 50−60% of both **4** and **5** reacted at this site.

In parallel with the above experiment of glycan **4**, also, a sample (1.5 pmol) of the fucose-containing heptasaccharide Galβ1−4GlcNAcβ1−3Galβ1−4([^{14}C]Fucα1−3)GlcNAcβ1−3Galβ1−4GlcNAc (**6**) was incubated with UDP-GlcNAc and the rat serum cIGnT6 activity. This incubation mixture revealed no formation of molecules larger than the acceptor. Only the single radiolabeled peak of the original glycan **6** (R_{MP} = 0.51; R_{MH} = 1.06) was observed in the paper chromatogram of the reaction mixture (Figure 2C). This shows that the fucose-carrying GlcNAc in the middle of the acceptor **6** prevented the branching reaction at the middle LacNAc and also at the adjacent upstream LacNAc, confirming the data obtained with glycans **2** and **3**.

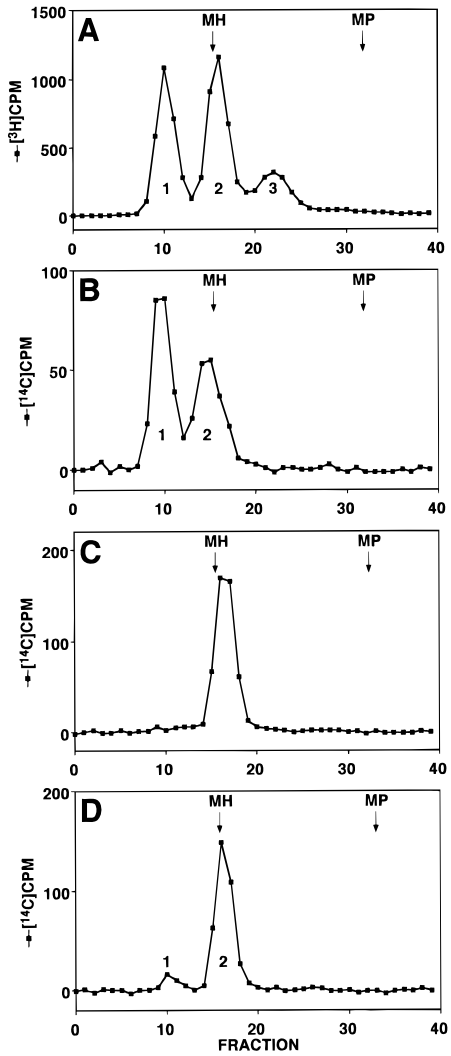


FIGURE 2: Paper chromatograms of oligosaccharides obtained when radiolabeled acceptor saccharides were incubated with UDP-GlcNAc and the cIGnT6 activity of rat serum. (Panel A) Transfer of β1,6-GlcNAc branches to the fucose-free hexasaccharide [^{3}H]-Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc (**4**). Solvent A, 329 h. Peak 1 represents the doubly branched octasaccharide **11**, peak 2 is a mixture of the heptasaccharides **9** and **10**, and peak 3 contains the unreacted acceptor **4**. (Panel B) Branching of Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4([^{14}C]-Fucα1−3)GlcNAc (**5**). Solvent A, 329 h. Peak 1 represents the branched octasaccharide **12** and peak 2 is the remaining heptasaccharide acceptor **5**. (Panel C) Attempted branching of Galβ1−4GlcNAcβ1−3Galβ1−4([^{14}C]Fucα1−3)GlcNAcβ1−3Galβ1−4GlcNAc (**6**). Solvent A, 329 h. Only the acceptor is detected. (Panel D) Branch formation in Galβ1−4([^{14}C]Fucα1−3)GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc (**7**). Solvent A, 329 h. Peak 1 represents the branched octasaccharide **13** while peak 2 is the unreacted acceptor **7**.

A third branch-forming reaction, also carried out in parallel with the experiment of glycan **4**, was performed with 1.5 pmol of the fucose-containing heptasaccharide Galβ1−4([^{14}C]Fucα1−3)GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc (**7**). The products of this reaction revealed a small amount (9%) of an octasaccharide-like glycan ($R_{MP} = 0.31$; $R_{MH} = 0.65$) together with a large peak of the unreacted acceptor ($R_{MP} = 0.49$; $R_{MH} = 1.02$) (Figure 2D). This experiment was repeated, yielding similar data. *endo-β*-Galactosidase digestion cleaved 75% of the octasaccharide-like product at the middle galactosidic linkage, liberating Galβ1−4([^{14}C]Fucα1−3)GlcNAcβ1−3Gal (Figure 3). The

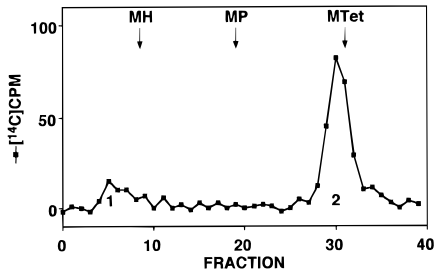


FIGURE 3: Paper chromatogram of oligosaccharides of an *endo-β*-galactosidase digest from the octasaccharide Galβ1−4([^{14}C]-Fucα1−3)GlcNAcβ1−3Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)-Galβ1−4GlcNAc (**13**). Peak 2 represents Galβ1−4([^{14}C]Fucα1−3)GlcNAcβ1−3Gal formed by a cleavage at the middle LacNAc of the substrate. Solvent A, 240 h.

data imply that most of the slow GlcNAc transfer in glycan **7** had occurred at the galactose of the reducing end LacNAc, forming the glycan **13**. Hence, even in glycan **7**, the fucose unit had prevented the branch-forming reaction at the adjacent upstream LacNAc unit.

Central Branching Was Prevented Even at an Upstream Galactose, Distant from the Fucosylated GlcNAc Residue. The effects of α1,3-bonded fucose units of polylactosamine chains on the branching potential of even a distantly located galactose site could be estimated from the data of Figure 2. The parallel branching experiments of the heptasaccharide **5** (Figure 2B) and the hexasaccharide **4** (Figure 2A) revealed that the middle galactoses of **5** and **4** reacted in about 55 and 63% of the molecules, respectively. Obviously, the branching potentials of **4** and **5** were approximately equal at the middle galactose units, the fucosyl residue in glycan **5** being no significant branching inhibitor at the middle LacNAc.

In contrast to glycan **5**, the branching reaction of glycan **7**, having the fucosyl residue at the nonreducing end LacNAc, resulted in the formation of an octasaccharide-like fraction only with a small yield of about 9% (Figure 2D). In the control experiment, about 60% of the fucose-free acceptor **4** had reacted at the reducing end LacNAc unit as judged from the yields of the octa- and heptasaccharide products in Figure 2A and from the reported composition of the heptasaccharide fraction obtained under these conditions (Leppänen et al., 1997). Hence, the fucosyl residue at the nonreducing end LacNAc unit inhibited almost 90% of the branching reaction of **7** at the reducing end LacNAc, at a site separated from the fucosylated locus by two monosaccharide residues. This unexpected result was confirmed in a repeated experiment similar to that of Figure 2D.

DISCUSSION

The present observations describe midchain branching of fucose-containing polylactosamines. The unique acceptor oligosaccharides were synthesized enzymatically in our laboratory (Niemelä et al., 1995c; R., Niemelä, et al., unpublished experiments) and were used in the β1,6-GlcNAc transferase experiments in tracer amounts under conditions where the fucose-free analogues reacted to the extent of 60−80%. The branching reactions under study were catalyzed by the β1,6-GlcNAc transferase (cIGnT6) activity, which is present in the blood serum of man and rat and reacts at midchain positions of the acceptors (Leppänen et al., 1991, 1997; Gu et al., 1992).

We show here that enzymatic *in vitro* synthesis of backbone branches is strongly restricted in α1,3-fucosylated polylactosamine chains. In particular, reactions like Gal*β*1−4GlcNAc*β*1−3[^{14}C]Gal*β*1−4GlcNAc (**1**) + UDP-GlcNAc → Gal*β*1−4GlcNAc*β*1−3(GlcNAc*β*1−6)[^{14}C]Gal*β*1−4GlcNAc (**8**) + UDP were readily catalyzed by the centrally acting *β*1,6-GlcNAc transferase activities (cIGnT6) of mammalian sera, but were not observed when α1,3-fucosylated derivatives of glycan **1**, i.e., Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4([^{14}C]Fucα1−3)GlcNAc (**2**) or the isomeric Gal*β*1−4([^{14}C]-Fucα1−3)GlcNAc*β*1−3Gal*β*1−4GlcNAc (**3**), were used as acceptors.

The observation that α1,3-fucosylation at either of the adjoining GlcNAc residues of the acceptor site prevents the branch formation completely was not unexpected, because α1,3-fucosylation is known to be frequently associated with marked changes in the properties of *N*-acetyllactosamine saccharides. In particular, sugar−protein interactions are remarkably affected by α1,3- fucosylation; reported examples include saccharide interactions with *exo*-*β*-galactosidases (Arakawa et al., 1974), α2,3-sialyltransferases (Weinstein et al., 1982), *β*1,3-GlcNAc-transferases (Piller & Cartron, 1983), *Datura stramonium* agglutinin (Yamashita et al., 1987), wheat germ agglutinin (Renkonen et al., 1991a; Niemelä et al., 1995a), *endo*-*β*-galactosidases (Nakagawa et al., 1980; Kannagi et al., 1982; de Vries et al., 1993), and E-, P- and L-selectins (Lasky, 1995). Hence, our present data simply add another enzyme, the cIGnT6 activity of human and rat serum, to the growing list of proteins that bind differently to fucose-free and α1,3-fucosylated *N*-acetyllactosamine saccharides.

Several covalent and noncovalent changes resulting from the fucosylation may be responsible for the observed unreactivity of glycans **2** and **3** with cIGnT6. (i) The hydroxyl groups at 3-positions of the GlcNAc residues, which may be crucial for the proper formation of enzyme-substrate complex, become substituted in the fucosylation reaction. (ii) In the distal Gal*β*1−4(Fucα1−3)GlcNAc*β*1-OR sequence of glycan **3**, fucose is believed to mask intramolecularly some elements of the galactose (Wormald et al., 1991; Ball et al., 1992; Ichikawa et al., 1992; Miller et al., 1992), which may also be crucial for the substrate binding to cIGnT6. (iii) Even in the reducing end Lewis x sequence of glycan **2**, the galactose is probably masked to some extent by the fucose; the stable conformation of Lewis x is not perturbed by *N*-acetylneuraminic acid substitution at position 3 of the galactose unit (Ball et al., 1992), and it may tolerate analogous substitution by LacNAc as well.

We have recently been able to obtain isomeric monofucosylated derivatives in pure form also from the hexasaccharide Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4GlcNAc (**4**) (R. Niemelä, et al., unpublished experiments). Using the heptasaccharide Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4([^{14}C]Fucα1−3)GlcNAc*β*1−3Gal*β*1−4GlcNAc (**6**) as a substrate for cIGnT6 activity of rat serum, we could not observe any branching reaction, although the nonfucosylated glycan **4** reacted readily at both internal galactoses. This finding confirmed the data obtained with glycans **2** and **3**.

By using the heptasaccharide Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4(Fucα1−3)GlcNAc (**5**) as the substrate for serum cIGnT6 activity, we found no branching at the fucosylated LacNAc unit, but at the middle LacNAc, the branching occurred as readily as in the fucose-free glycan **4**. Hence, the fucose of glycan **5** did not exert its inhibitory effect towards the nonreducing end from the fucosylated LacNAc unit. By contrast, the heptasaccharide Gal*β*1−4(Fucα1−3)GlcNAc*β*1−3Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4GlcNAc (**7**) did not react with cIGnT6 at the middle LacNAc and reacted poorly even at the reducing end LacNAc, two intact monosaccharides away toward the reducing end from the fucosylated site. At this site, we had expected a fully efficient reaction, because there is a complete sequence Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4GlcNAc in the reducing end area of glycan **7**, and this sequence usually reacts well with cIGnT6. In particular, our previous experiments (Leppänen et al., 1997) as well as the present data show that, in the fucose-free glycan Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4GlcNAc (**4**), the reducing end LacNAc reacts readily with the cIGnT6. To explain the low reactivity of glycan **7**, we suggest that the distal Lewis x determinant probably interacts with some elements of the molecule's proximal Gal*β*1−4GlcNAc*β*1−3Gal*β*1−4GlcNAc epitope in a way that prevents the normal binding of this determinant to cIGnT6.

On a practical level, the present data on selective branch-formation in α1,3-fucosylated polylactosamines make site-directed branch genesis by cIGnT6 possible *in vitro*, because α1,3-fucosyl units can be positioned in linear polylactosamines to direct branch formation (R. Niemelä, et al., unpublished experiments) and afterward they can be removed without harming the backbones (Hounsell et al., 1985).

Quite recent experiments show in our laboratory that lysates of human embryonal carcinoma cells of line PA1 contain *β*1,6-GlcNAc transferase activity that catalyzes branch formation at linear polylactosamines and resembles closely the serum cIGnT6 activity in its properties (A. Leppänen, et al., unpublished experiments). This is highly relevant, because the PA-1 cells express very large amounts of highly branched polylactosamines (Rasilo & Renkonen, 1982; Fukuda et al., 1985), suggesting that the cIGnT6 activity of the lysates is closely associated with the biosynthesis of polylactosamine branches *in vivo*. Thus, the midchain branch formation of polylactosamines is probably incompatible with preceeding α1,3-fucosylation *in vivo*, too.

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