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Biosynthesis of Branched Polylactosaminoglycans

EMBRYONAL CARCINOMA CELLS EXPRESS MIDCHAIN β1,6-*N*-ACETYLGLUCOSAMINYLTRANSFERASE ACTIVITY THAT GENERATES BRANCHES TO PREFORMED LINEAR BACKBONES*

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Two types of β1,6-GlcNAc transferases (IGnT6) are involved in *in vitro* branching of polylactosamines: dIGnT6 (distally acting), transferring to the penultimate galactose residue in acceptors like GlcNAcβ1–3*Gal*β1–4GlcNAcβ1-R, and cIGnT6 (centrally acting), transferring to the midchain galactoses in acceptors of the type (GlcNAcβ1–3)Galβ1–4GlcNAcβ1–3*Gal*β1–4GlcNAcβ1-R. The roles of the two transferases in the biosynthesis of branched polylactosamine backbones have not been clearly elucidated. We report here that cIGnT6 activity is expressed in human (PA1) and murine (PC13) embryonal carcinoma (EC) cells, both of which contain branched polylactosamines in large amounts. In the presence of exogenous UDP-GlcNAc, lysates from both EC cells catalyzed the formation of the branched pentasaccharide Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc from the linear tetrasaccharide Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc. The PA1 cell lysates were shown to also catalyze the formation of the branched heptasaccharides Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc and Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc from the linear hexasaccharide Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc in reactions characteristic to cIGnT6. By contrast, dIGnT6 activity was not detected in the lysates of the two EC cells that were incubated with UDP-GlcNAc and the acceptor trisaccharide GlcNAcβ1–3Galβ1–4GlcNAc. Hence, it appears likely that cIGnT6, rather than dIGnT6 is responsible for the synthesis of the branched polylactosamine chains in these cells.

Biosynthesis of branched backbones of type 2 polylactosamines involves reactions catalyzed by β1,3-*N*-acetylglucosaminyltransferase (GnT3),[1] β1,4-galactosyltransferase (GalT4), and β1,6-*N*-acetylglucosaminyltransferases (GnT6). However, the actual pathways leading to *in vivo* biosynthesis of branched polylactosamine backbones have not been clearly identified. The branch-forming reactions, in particular, are poorly understood. Two candidate branching reactions involving distinct β1,6-*N*-acetylglucosaminyltransferases (IGnT6) have been described *in vitro* (1). The "distally acting" dIGnT6 transfers a GlcNAc unit in the β1,6 linkage to the penultimate galactose residue at the growing end of the linear polylactosamine chain (Scheme 1) (2–8). By contrast, the "centrally acting" cIGnT6 transfers a GlcNAc residue in the β1,6 linkage to midchain galactose units of preformed as well as growing linear chains (3, 9, 10). The dIGnT6 reactions proceed only with acceptor chains bearing distal GlcNAc residues, whereas the cIGnT6 works with polylactosamine backbones carrying either a galactose or a GlcNAc residue at the distal position. There is very little overlapping in the acceptor specificities of the two types of enzymes *in vitro*.

In naturally occurring branched backbones, uniformly short LacNAcβ1–6 branches are linked to linear primary chains; this is the case *e.g.* in human embryonal carcinoma (EC) cells (11) and adult erythrocyte band 3 (12). It has been suggested that the dIGnT6 is responsible for the biosynthesis of polylactosamines in these cells (5, 11, 12). However, the suggested role of dIGnT6 in the biosynthesis of molecules containing exclusively short branches is doubtful because the extension enzyme, *e.g.* the GnT3 of human serum elongates both branches of the hexasaccharide LacNAcβ1–3′(LacNAcβ1–6′)LacNAc (where LacNAc is Galβ1–4GlcNAc) (13), paving routes to the formation of complex as well as short branches (14).

We have recently suggested that midchain branching enzymes similar to the cIGnT6 activity present in blood serum of mammals may be responsible for the conversion of linear polylactosamine chains into branched backbone arrays *in vivo* (10). The reasoning was based on data showing that the cIGnT6 activity of rat serum catalyzed the transformation of the linear hexasaccharide LacNAcβ1–3LacNAcβ1–3LacNAc in two steps into the doubly branched octasaccharide LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(GlcNAcβ1–6′)LacNAc. The latter was β1,4-galactosylated enzymatically into the mature decasaccharide backbone LacNAcβ1–3′(LacNAcβ1–6′)LacNAcβ1–3′(LacNAcβ1–6′)LacNAc (10), which strikingly resembles the polylactosamine backbones of human EC cells of line PA1 (11).

Here, we report experiments involving lysates of human PA1 cells, exogenous UDP-GlcNAc, and either the tetrasaccharide

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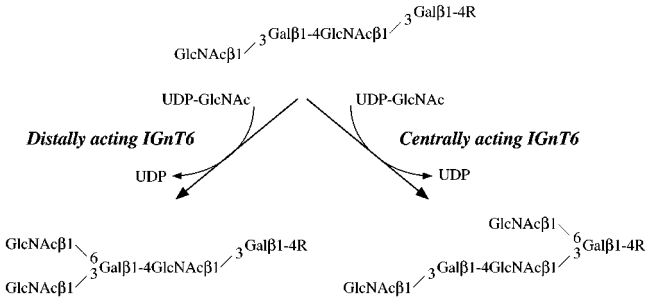
[1] The abbreviations used are: GnT3, β1,3-*N*-acetylglucosaminyltransferase (GlcNAc to Gal); GalT4, β1,4-galactosyltransferase (Gal to GlcNAc); cIGnT6, centrally acting β1,6-*N*-acetylglucosaminyltransferase (GlcNAc to Gal); dIGnT6, distally acting β1,6-*N*-acetylglucosaminyltransferase (GlcNAc to Gal); EC, embryonal carcinoma; Gal (or G), D-galactose; GlcNAc (or Gn), *N*-acetyl-D-glucosamine; Lac, lactose; LacNAc, *N*-acetyllactosamine (Galβ1–4GlcNAc); MALDI-TOF mass spectroscopy, 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].



SCHEME 1. **Formation of GlcNAc branches at different positions of a linear polylactosaminoglycan by the two types of IGnT6s (2–10).**

LacNAcβ1–3′LacNAc or the hexasaccharide LacNAcβ1–3′-LacNAcβ1–3′LacNAc that established the presence of the cIGnT6 activity in the PA1 cells. In contrast, the dIGnT6 activity was not detected in experiments where UDP-GlcNAc and GlcNAcβ1–3Galβ1–4GlcNAc were incubated with PA1 cell lysates. Lysates of murine EC cells of line PC13, known to carry large amounts of branched polylactosamines (15), also expressed the cIGnT6 activity but not the dIGnT6 activity. The data imply that cIGnT6 rather than dIGnT6 activity is involved in the biosynthesis of branched polylactosaminoglycans in human as well as murine EC cells. Hence, it is suggested that the linear polylactosamine backbones are probably synthesized first and branched afterward in these cells.

EXPERIMENTAL PROCEDURES

Cells—Mouse embryonal carcinoma cells of line PC13 established from the pluripotent OTT6050 teratocarcinoma tumor (16) were obtained from Dr. C. F. Graham (Department of Zoology, University of Oxford, UK). The human PA1 teratocarcinoma-derived cells (17) were obtained from Dr. Jorma Wartiovaara (Institute of Biotechnology, University of Helsinki, Finland). The cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum as described (18). For the experiments, the cells were detached from the dishes with 0.02% EDTA in NaCl-P buffer (140 mM NaCl, 10 mM sodium phosphate, pH 7.2) and washed twice in Dulbecco's phosphate-buffered saline, pH 7.2–7.4 (with Ca^{2+} and Mg^{2+}).

Preparation of Cell Lysates—Washed human (PA1) and mouse (PC13) EC cell pellets (50–150 μl) were lysed with 200 μl of 0.9% NaCl, 1% TX-100, 1 mM phenylmethylsulfonyl fluoride. In some experiments, more concentrated cell lysates were prepared by suspending the cell pellets in 50 μl of 1.8% NaCl, 2% TX-100, 2 mM phenylmethylsulfonyl fluoride with the cells. Small amounts (0.6 μl) of 40 mM phenylmethylsulfonyl fluoride in ethanol were added to the mixture once each h to a final concentration of 1 mM. In some experiments aprotinin and leupeptin were also added to the lysis buffer to a final concentration of 17 μg/ml and 20 μg/ml, respectively. The lysed cells were kept at 0 °C and homogenized by 5 × 3 strokes in a Potter homogenizer. The lysates were used immediately as the enzyme source in glycosyltransferase reactions.

Acceptor Oligosaccharides—The acceptor oligosaccharides (for the structures see also Table I) were synthesized as described: LacNAcβ1–3[^{14}C]Galβ1–4GlcNAc (**1**) (9), unlabeled **1** (19); two isotopomers of hexasaccharide **3** (LacNAcβ1–3[^{3}H]Galβ1–4GlcNAcβ1–3′LacNAc and LacNAcβ1–3′LacNAcβ1–3[^{14}C]Galβ1–4GlcNAc) (10); [^{14}C]GlcNAcβ1–3′LacNAc (**7**) (7). A (1:1)-mixture of [^{3}H]heptasaccharides **4** and **5** was synthesized from [^{3}H]Galβ1–4GlcNAcβ1–3′LacNAcβ1–3′LacNAc (**3**) as described in Leppänen *et al.* (10).

Marker Oligosaccharides—The following radiolabeled marker oligosaccharides were synthesized as described: **7**, **8**, and GlcNAcβ1–6′-LacNAc (7); LacNAcβ1–3Gal (9); **2** (19); LacNAcβ1–3′(LacNAcβ1–6′)-LacNAc (13); a mixture of the heptasaccharides [^{3}H]Galβ1–4GlcNAcβ1–3LacNAcβ1–3′(GlcNAcβ1–6′)LacNAc (**4**) + [^{3}H]Galβ1–4GlcNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′LacNAc (**5**) and the octasaccharide LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(GlcNAcβ1–6′)LacNAc (10). [^{3}H]Galβ1–4GlcNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3Gal was obtained by endo-β-galactosidase cleavage of authentic appropriately radiolabeled heptasaccharide **5** (10). The octasaccharide **6** (R_{MP} = 0.29, R_{MH} = 0.63; solvent A) was synthesized by β1,4-galactosylating the hexasaccharide GlcNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′LacNAc (8).

Glycosyltansferase Reactions—The cIGnT6 reactions were performed by incubating the acceptors (3 pmol–100 nmol) and 3.7 μmol of UDP-GlcNAc with 25 μl of the EC cell lysate for 4 h and in some cases for 21–23 h in a total volume of 25 μl of 50 mM Tris-HCl buffer, pH 7.5, 8 mM NaN_3, 20 mM EDTA, 0.5 mM ATP, 20 mM D-galactose, 60 mM γ-galactonolactone, and 100 mM GlcNAc. EDTA inhibited the serum GnT3 activity (20), D-galactose and γ-galactonolactone were added to inhibit β-galactosidase activity, and GlcNAc was used to inhibit β-N-acetylhexosaminidase activity. The dIGnT6 reactions with the teratocarcinoma cell lysates were carried out essentially as described for hog gastric mucosal microsomes (7), but incubation times of 4 h were used, and the total volume of the reaction mixture was 25 μl. All IGnT6 reaction mixtures were passed through a mixed bed of Dowex AG1 (AcO^-) and Dowex AG 50 (H^+), and the eluates were lyophilized.

Galactosylation with bovine milk β1,4-galactosyltransferase (EC 2.4.1.90) (Sigma) was performed essentially as described in Brew *et al.* (21).

Chromatographic Methods—Paper chromatographic runs of desalted radiolabeled saccharides were performed on Whatman III Chr paper with the upper phase of 1-butanol/acetic acid/water (4:1:5 v/v; solvent A) or with 1-butanol/ethanol/water (10:1:2 v/v, solvent B). Radioactivity on the chromatograms was monitored as in Leppänen *et al.* (10) using Optiscint (Wallac, Turku, Finland) as scintillant. Marker lanes of malto-oligosaccharides on both sides of the sample lanes were stained with silver nitrate.

Gel permeation chromatography on a column of Superdex 75 HR 10/30 or Superdex Peptide HR 10/30 (Amersham Pharmacia Biotech) was performed as in Niemelä *et al.* (19).

Degradative Experiments—Digestions with endo-β-galactosidase from *Bacteroides fragilis* (EC 3.2.1.103) (Boehringer Mannheim) were performed according to Leppänen *et al.* (9); parallel control reactions cleaved over 90% of radiolabeled GlcNAcβ1–3Galβ1–4GlcNAc.

Digestions with jack bean β-galactosidase (EC 3.2.1.23) were carried out as described in Renkonen *et al.* (22). Partial digestions with jack bean β-*N*-acetylhexosaminidase (EC 3.2.1.30) were performed as in Leppänen *et al.* (10).

Partial acid hydrolysis was carried out using 0.1 M trifluoroacetic acid at 100 °C essentially as in Seppo *et al.* (7).

^{1}H NMR Experiments—The ^{1}H NMR experiments were carried out as in Niemelä *et al.* (19).

Matrix-assisted Laser Desorption/Ionization Mass Spectrometry—Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed in the positive-ion delayed-extraction mode with a BIFLEX™ mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) using a 337-nm nitrogen laser. 1 μl of sample (10 pmol) and 1.5 μl of 2,5-dihydroxybenzoic acid matrix (10 mg/ml in water) were mixed on the target plate and dried with a gentle stream of air. Dextran standard 5000 from *Leuconostoc mesentroides* (Fluka Chemica-Biochemica) was used for external calibration.

RESULTS

The structures of key oligosaccharides of the present experiments are shown in Table I and are identified in the text by using appropriate bold face digits.

*Branching Reactions of Tetrasaccharide **1**, Catalyzed by Lysates of PA1 and PC13 Cells, Gave Pentasaccharide **2***—Incubation of tetrasaccharide Galβ1–4GlcNAcβ1–3[^{14}C]Galβ1–4GlcNAc (**1**) and UDP-GlcNAc with lysates of human embryonal carcinoma cells (line PA1) gave an oligosaccharide product that chromatographed on paper like authentic pentasaccharide **2** marker (peak 1 in Fig. 1*A*). Yields of 3–7% were obtained. The identity of the product as glycan **2** was established by enzymatic degradation. First, a treatment of the pentasaccharide with β-galactosidase gave a product co-chromatographing with authentic GlcNAcβ1–3(GlcNAcβ1–6)[^{14}C]Galβ1–4GlcNAc (**8**) (Fig. 1*B*). Next, the putative glycan **8** was identified by a partial treatment with β-*N*-acetylhexosaminidase that gave the trisaccharides GlcNAcβ1–6[^{14}C]Galβ1–4GlcNAc and GlcNAcβ1–3[^{14}C]Galβ1–4GlcNAc as well as the disaccharide [^{14}C]Galβ1–4GlcNAc (Fig. 1*C*). In addition to degradation, the original pentasaccharide product was subjected to MALDI-TOF mass spectrometry that gave a major signal at *m/z* 974.8 (Fig. 1*D*), assigned to the sodiated molecular ion of $Gal_2GlcNAc_3$ (calculated *m/z* = 974.9). Finally, the identity of the pentasaccharide

TABLE I
Structures of the key saccharides

No	Saccharide
1	Galβ1–4GlcNAcβ1 ⟋ $_{3}$Galβ1–4GlcNAc
2	GlcNAcβ1 ⟍ / Galβ1–4GlcNAcβ1 ⟋ $^{6}_{3}$Galβ1–4GlcNAc
3	Galβ1–4GlcNAcβ1 ⟋ $_{3}$Galβ1–4GlcNAcβ1 ⟋ $_{3}$Galβ1–4GlcNAc
4	Galβ1–4GlcNAcβ1 ⟋ $_{3}$Galβ1–4GlcNAcβ1 ⟋ GlcNAcβ1 ⟍ / $^{6}_{3}$Galβ1–4GlcNAc
5	GlcNAcβ1 ⟍ / Galβ1–4GlcNAcβ1 ⟋ $^{6}_{3}$Galβ1–4GlcNAcβ1 ⟋ $_{3}$Galβ1–4GlcNAc
6	Galβ1–4GlcNAcβ1 ⟍ / Galβ1–4GlcNAcβ1 ⟋ $^{6}_{3}$Galβ1–4GlcNAcβ1 ⟋ $_{3}$Galβ1–4GlcNAc
7	GlcNAcβ1 ⟋ $_{3}$Galβ1–4GlcNAc
8	GlcNAcβ1 ⟍ / GlcNAcβ1 ⟋ $^{6}_{3}$Galβ1–4GlcNAc

product generated by PA1 cell lysates was confirmed by the ^{1}H NMR spectrum (Fig. 1*E*, Table II); the resonances of the structural reporter groups were practically identical with those of authentic glycan **2** (23). Some of these resonances probably would have been different if the GlcNAc branch had been transferred to C-2 or C-4 of the central galactose unit of glycan **1** (24).

The pentasaccharide **2** was formed from tetrasaccharide **1** in yields of 6–10%, also in reactions catalyzed by lysates of murine EC cells of line PC13 (Fig. 2*A*). The endo-β-galactosidase-resistant product was identified as glycan **2** by enzymatic degradation as above. First, β-galactosidase converted the putative pentasaccharide **2** into a tetrasaccharide that chromatographed like glycan **8** (Fig. 2*B*). Then the putative glycan **8**, upon a partial treatment with β-*N*-acetylhexosaminidase gave a mixture of the trisaccharides GlcNAcβ1–6[^{14}C]Galβ1–4GlcNAc and GlcNAcβ1–3[^{14}C]Galβ1–4GlcNAc as well as the disaccharide [^{14}C]Galβ1–4GlcNAc (Fig. 2*C*). Finally, enzymatic β1,4-galactosylation of the putative pentasaccharide **2** gave the hexasaccharide LacNAcβ1–3′(LacNAcβ1–6′)LacNAc (Fig. 2*D*), establishing the presence of a distal GlcNAc in the acceptor.

*Branching Reactions of Hexasaccharide **3**, Catalyzed by PA1 Cell Lysates Gave Heptasaccharide Isomers **4** and **5***—Two isotopomers of glycan **3** (LacNAcβ1–3′LacNAcβ1–3[^{14}C]Galβ1–4GlcNAc and LacNAcβ1–3[^{3}H]Galβ1–4GlcNAcβ1–3′LacNAc) were synthesized and were separately incubated with PA1 cell lysates and UDP-GlcNAc to establish whether both the galactose 2 (labeled in the [^{14}C]-acceptor) and the galactose 4 (labeled in the [^{3}H]-acceptor) of glycan **3** serve as independent acceptor sites. A heptasaccharide-like product was formed from both acceptors. The product chromatographed as a single peak (peak *1* in Fig. 3*A*, R_{MP} = 0.49, R_{MH} = 1.04, solvent A), showing the same migration rate as an unresolved mixture of authentic heptasaccharides **4** and **5** (10). The net yield of the heptasaccharide-like fraction varied from 3 to 7% in 4-h incubations in several separate experiments; it was not improved in 22-h incubations. To ensure that peak 1/Fig. 3*A* represented an authentic product resulting from the transfer of GlcNAc to radiolabeled acceptor **3**, an aliquot of the material was treated with UDP-Gal and GalT4. This treatment gave 62% [^{3}H]glycans chromatographing like authentic octasaccharide **6** (Fig. 3*B*, R_{MP} = 0.32, R_{MH} = 0.68, solvent A), establishing the presence of a distal GlcNAc residue in most of the glycans of peak *1*/Fig. 3*A*. In addition, the data of Fig. 3*B* suggest that the glycans of peak *1*/Fig. 3*A* also included the [^{3}H]hexasaccharide **3** acceptor itself, which contaminated the heptasaccharide products because of obvious chromatographic "tailing." Other experiments that are described below show that the distal GlcNAc units of the heptasaccharide products of the branching reaction of glycan **3** were β1,6-bonded in some molecules to the galactose 2 and in others to the galactose 4 of the acceptor.

The presence of glycan **4** in the heptasaccharide fraction of the branching reaction was established by using endo-β-galactosidase, which cleaves internal β-galactosidic linkages of polylactosamines but does not attack these linkages at branch-bearing *N*-acetyllactosamine units (25). Treatment of the crude [^{14}C]heptasaccharide fraction with this enzyme gave two labeled oligosaccharide products, shown in Fig. 3*C*. Peak *2*, chromatographing like the branched tetrasaccharide **8**, was derived from the putative glycan **4**. It was isolated and cleaved by partial β-*N*-acetylhexosaminidase treatment into GlcNAcβ1–6[^{14}C]Galβ1–4GlcNAc (peak *2*) and GlcNAcβ1–3[^{14}C]Galβ1–4GlcNAc (peak *3*) as shown in Fig. 3*D*. These data established that during the incubation with UDP-GlcNAc and PA1 cell lysate, a β1,6-bonded GlcNAc branch had been transferred to galactose 2 of the [^{14}C]-labeled glycan **3**. Peak *1* of Fig. 3*C* represented the hexasaccharide LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ3[^{14}C]Gal, a cleavage product of glycan **5**; it was not studied further because it contained no easily accessible structural information about the branch structure.

The presence of glycan **5** in the crude [^{3}H]heptasaccharide fraction of the branching reaction was also established by using endo-β-galactosidase, which gave two radiolabeled oligosaccharides, LacNAcβ1–3[^{3}H]Gal (from glycan **4**) and LacNAcβ1–3-(GlcNAcβ1–6)[^{3}H]Galβ1–4GlcNAcβ1–3Gal, that were separated by paper chromatography (not shown). The hexasaccharide product, which was derived from the putative glycan **5** of the [^{3}H]heptasaccharide fraction, was then subjected to partial acid hydrolysis. This resulted in formation of several [^{3}H]oligosaccharides shown in Fig. 3*E*. The important products were GlcNAcβ1–6[^{3}H]Galβ1–4GlcNAc (peak *2*) and GlcNAcβ1–6[^{3}H]Gal (peak *4*), which proved that the galactose 4 of the [^{3}H]-labeled glycan **3** had accepted a β1,6-bonded GlcNAc branch during the incubation with UDP-GlcNAc and a PA1 cell lysate.

*The Branching Reaction of the Mixed Heptasaccharides **4** and **5**, Catalyzed by PA1 Cell Lysates, Gave an Octasaccharide*—Incubation of an authentic 1:1 mixture of [^{3}H]-labeled heptasaccharides **4** and **5** with UDP-GlcNAc and PA1 cell lysates gave small amounts (2.4–4.4%) of a product that chromatographed on paper like the doubly branched octasaccharide LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(GlcNAcβ1–6′)-LacNAc (not shown; R_{MP} = 0.74, R_{MH} = 1.10). In the MALDI-TOF mass spectrum the product gave a sodium-containing molecular ion at *m/z* 1543.4 (calculated *m/z* for $Gal_3HexNAc_5$ 1543.4). Together with the $(M + K)^+$ signal, this ion represented 70% of the total polylactosamines in the molecular ion range (26). A treatment of the octasaccharide concentrate with GalT4 and UDP-Gal gave a crude decasaccharide. In MALDI-TOF mass spectrum of this product, a major signal at *m/z*

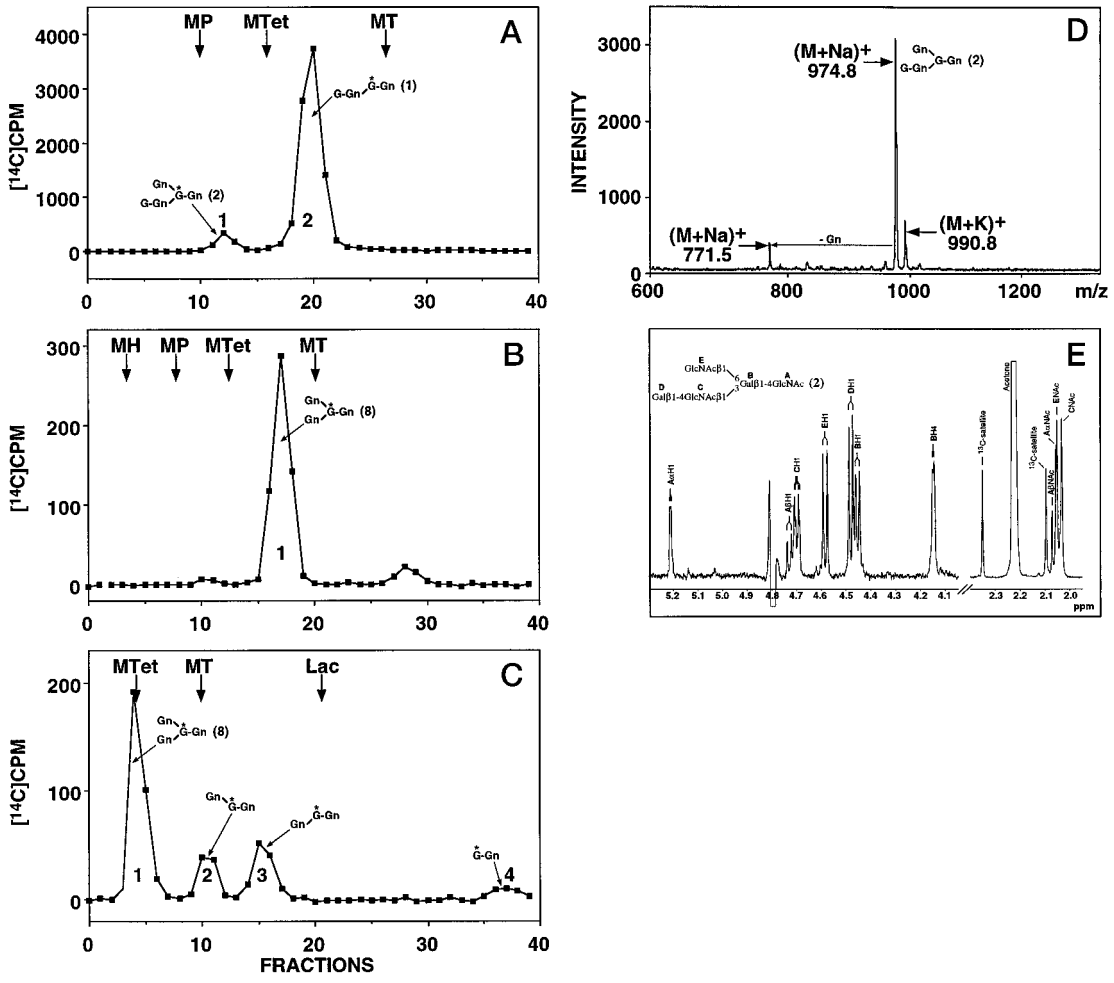


FIG. 1. **Product analysis of the branching reaction of tetrasaccharide 1 catalyzed by the cIGnT6 activity of PA1 cells.** *A*, paper chromatography (solvent A for 113 h) of the reaction mixture. Peak *1* represents the pentasaccharide product that chromatographed like authentic pentasaccharide **2**; peak *2* is the unreacted acceptor **1**. Unlabeled markers MT, MTet, MP, (MH), maltotriose, -tetraose, -pentaose (and -heptaose), respectively. The position of the radiolabel in the glycans is shown by an *asterisk*. *B*, paper chromatography (solvent A for 96 h) of a β-galactosidase digest of peak *1* of *A*. The major product chromatographed like authentic tetrasaccharide **8**. *C*, paper chromatography (solvent B for 305 h) of a partial β-*N*-acetylhexosaminidase digest of peak *1* of *B*. Peak *1* chromatographed like uncleaved **8**, peak *2* like GlcNAcβ1–6[^{14}C]Galβ1–4GlcNAc, peak *3* like GlcNAcβ1–3[^{14}C]Galβ1–4GlcNAc (**7**), and peak *4* like [^{14}C]Galβ1–4GlcNAc. Unlabeled markers are as in *A*. *D*, MALDI-TOF mass spectrum of the original pentasaccharide product. The signal at *m/z* 974.8 was assigned to $(M + Na)^+$ of $Gal_2GlcNAc_3$ (calculated *m/z* 974.9). The signal at *m/z* 990.8 was assigned to $(M + K)^+$ (calculcated *m/z* 990.3). *E*, reporter group signals of a 500-MHz ^{1}H NMR spectrum of the original pentasaccharide product (20.5 nmol) at 23 °C; the *vertical scale* at 4.1–5.3 ppm is multiplied by 4. The spectrum was very nearly identical with the spectrum of authentic pentasaccharide **2** generated by the cIGnT6 of rat serum (23) (see Table II).

1867.8 was observed that was assigned to $(M + Na)^+$ of $Gal_5HexNAc_5$ (calculated *m/z* 1867.7). These data suggest that the octasaccharide formed from the mixture of the heptasaccharides **4** and **5** by the PA1 cell lysate was [^{3}H]LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(GlcNAcβ1–6′)LacNAc.

Human (PA1) and Mouse (PC13) Embryonal Carcinoma Cells Did Not Reveal the Presence of the dIGnT6 Activity—When the trisaccharide [^{14}C]GlcNAcβ1–3Galβ1–4GlcNAc (**7**) (21 pmol) was incubated with UDP-GlcNAc and human or mouse EC cell lysates, chromatographically detectable tetrasaccharide-like products were formed in less than 0.3% yield (not shown), implying that human and mouse EC cells did not express significant dIGnT6 activities.

DISCUSSION

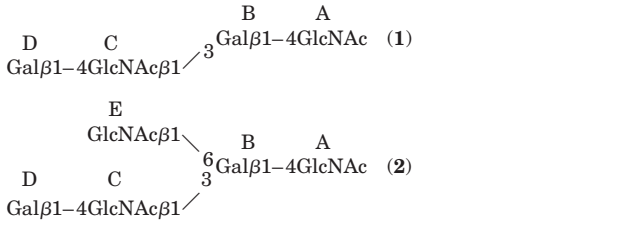
The present data show that lysates from human embryonal carcinoma cells of line PA1 contained centrally acting β1,6-*N*-acetylglucosaminyltransferase activity, which catalyzed the formation of the branched pentasaccharide **2** from the linear tetrasaccharide **1** in the presence of exogenous UDP-GlcNAc (For the structures of the saccharides, see Table I). In addition, heptasaccharides **4** and **5** were formed from the linear hexasaccharide **3**. Evidence was also provided supporting the notion that a second β1,6-GlcNAc branch was transferred during incubation of a mixture of the heptasaccharides **4** and **5** with PA1 cell lysates and UDP-GlcNAc. We call the activity responsible for these reactions as cIGnT6 to emphasize the site-specificity of the reaction in the *c*entral area of the acceptor and the formation of precursors of the blood group I antigen.

Because PA1 cells are known to express branched polylactosamine backbones (11, 27), it is reasonable to assume that the *in vitro* reactions described in the present experiments are similar to those responsible for the synthesis of the multiply branched polylactosamines *in vivo*.

Also lysates from murine embryonal carcinoma cells of line PC13 contained cIGnT6 activity. The PC13 cells are also known to express large amounts of branched polylactosamines (15). Hence, the cIGnT6 reactions observed *in vitro* in the present experiments are likely to occur also *in vivo* during the synthesis of PC13 glycans.

The action of dIGnT6, too, leads *in vitro* to the formation of

TABLE II
*^1H chemical shifts of tetrasaccharide **1** and pentasaccharide **2** at 23 °C in ^2H_2O*



Reporter group	Residue	Saccharide		
		1[a]	**2**[a]	**2**[b]
H-1	A[c]	5.205 (α)	5.212 (α)	5.212 (α)
		4.720 (β)	4.731 (β)	4.730 (β)
	B	4.464	4.454	4.453
	C[c]	4.706	4.701	4.701
		4.702	4.696	4.696
	D	4.479	4.481	4.480
	E		4.585	4.585[d]
H-4	B	4.158	4.149	4.148
NAc	A	2.040 (α)	2.056 (α)	2.055 (α)
		2.040 (β)	2.071 (β)	2.071 (β)
	C	2.035	2.032	2.032
	E		2.051	2.050

[a] From ^1H NMR spectra of authentic tetrasaccharide **1** and pentasaccharide **2** described by Maaheimo *et al.* (23).
[b] The pentasaccharide **2** was synthesized in the present experiments by using a PA1 cell lysate as the enzyme.
[c] The two values given correspond to the two anomers of the oligosaccharide.
[d] $^3J_{1,2}$ = 8.5 Hz.

branched polylactosamines (14), and this enzyme has been suggested to be responsible for the *in vivo* biosynthesis of branched polylactosamine backbones (5, 11, 12). However, in the present experiments we were unable to observe any dIGnT6 activity in lysates of PA1 cells or PC13 cells that would have converted the linear trisaccharide GlcNAcβ1–3Galβ1–4GlcNAc into the branched tetrasaccharide GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc. Taken together, our present data imply that the cIGnT6 activity rather than the dIGnT6 activity may be responsible for the *in vivo* synthesis of branched polylactosamine backbones in embryonal carcinoma cells.

The dominant role of cIGnT6 in the branch generation, combined with the data showing that the branches of glycans in PA1 cells are short along the entire backbone chain (11), suggests that the biosynthesis of branched polylactosamine backbones in PA1 cells occurs in rather distinct stages as shown in Scheme 2: First, alternating action of GnT3 and GalT4 elongates the linear backbone chains to their final size. Second, the linear backbones are branched by cIGnT6 at different sites along the chains. Third, the GlcNAc branches are finally galactosylated by GalT4. A process of this kind is likely to produce rather similar branches along the entire primary backbone chain. By contrast, participation of dIGnT6 in the branching process would generate branches in association with chain elongation, probably leading to more complex branches in the proximal than the distal parts of the mature backbones.

The concept that linear polylactosamine chains are precursors of the branched backbones is not new. The relationship was proposed already in 1979 when the developmentally regulated expression of small i (linear chains) and big I (branched backbones) as blood group antigens in human and bovine red blood cells was described (28, 29). The present data merely provide the underlying mechanism of the interconversion in EC cells. Scheme 2 suggests also that cIGnT6 is localized in the

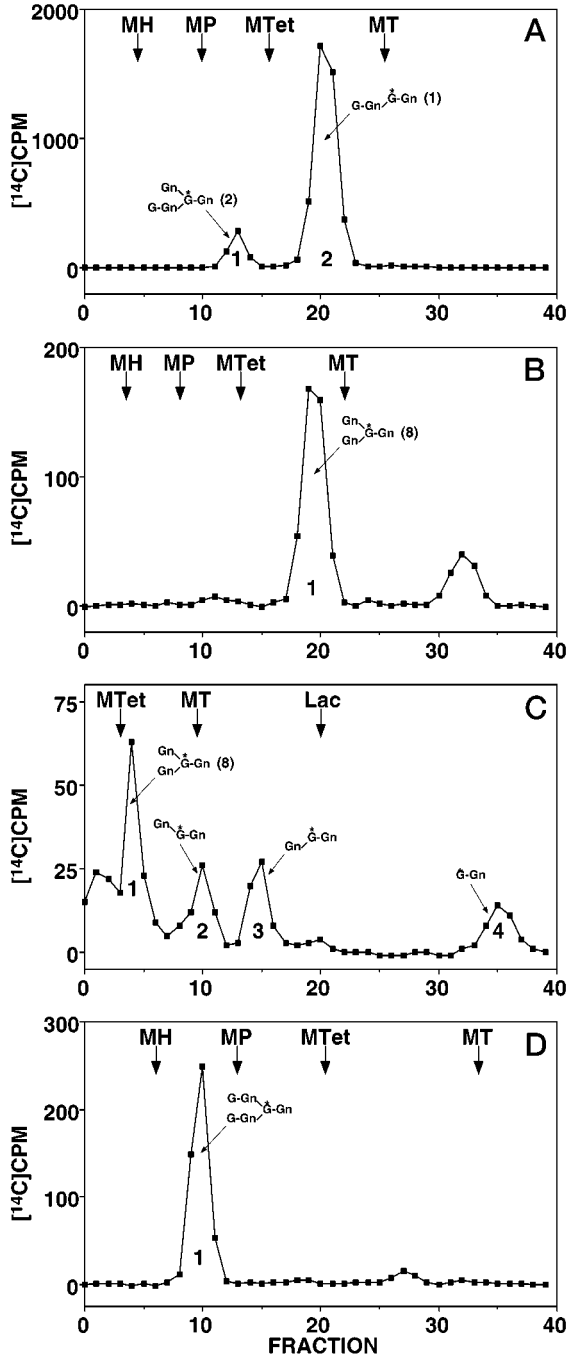


FIG. 2. **Product analysis of the branching reaction of tetrasaccharide 1 catalyzed by the cIGnT6 activity of PC13 cells.** *A*, paper chromatography (solvent A for 114 h) of the reaction mixture. Peak *1* represents the pentasaccharide product, and peak *2*, the unreacted acceptor **1**. The position of the radiolabel is shown by an *asterisk*. *B*, paper chromatography (solvent A for 113 h) of β-galactosidase digest of peak *1* of *A*. Peak *1* migrated like the marker tetrasaccharide **8**. *C*, paper chromatography (solvent B for 305 h) of partial β-*N*-acetylhexosaminidase digest of peak *1* of *B*. Peak *1* chromatographed like the uncleaved **8**, peak *2* like GlcNAcβ1–6[^{14}C]Galβ1–4GlcNAc, peak *3* like GlcNAcβ1–3[^{14}C]Galβ1–4GlcNAc, and peak *4* like [^{14}C]Galβ1–4GlcNAc. *D*, paper chromatography (solvent A for 141 h) of products from GalT4 reaction of peak *1* of *A*. Peak *1* chromatographed like authentic hexasaccharide LacNAcβ1–3′(LacNAcβ1–6′)LacNAc.

Golgi compartment of PA1 cells in a more restricted manner than Gal T4 and more distally than GnT3.

The presence of the cIGnT6 activity in the murine EC cell lysates suggests that the polylactosamine backbones of these cells may also consist of primary linear chains that carry short

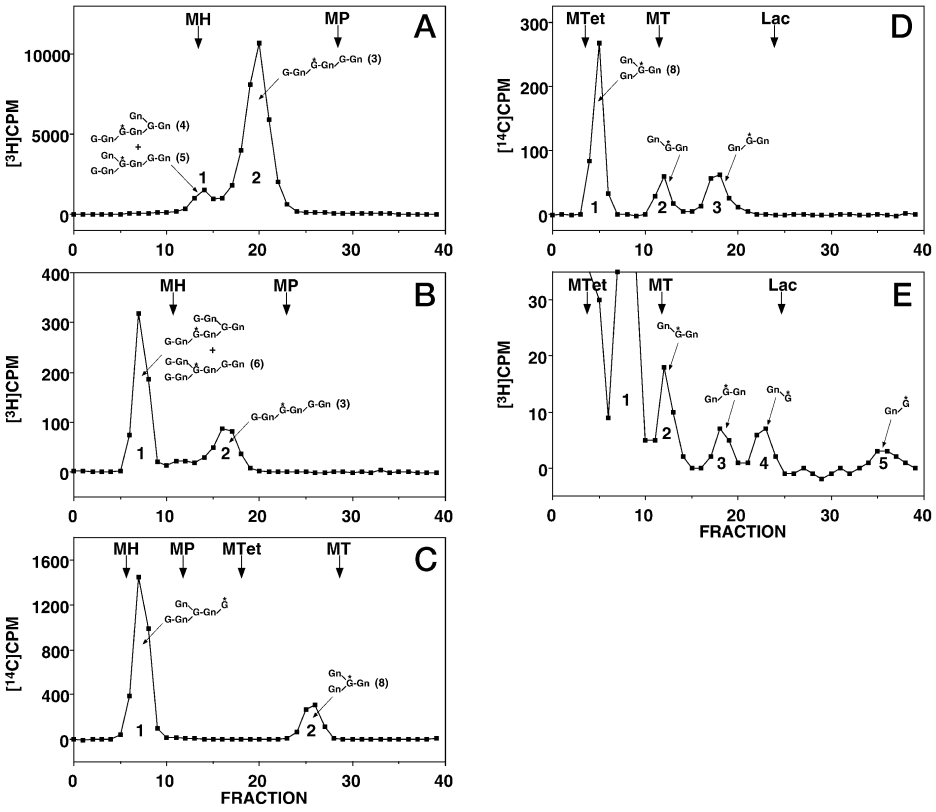
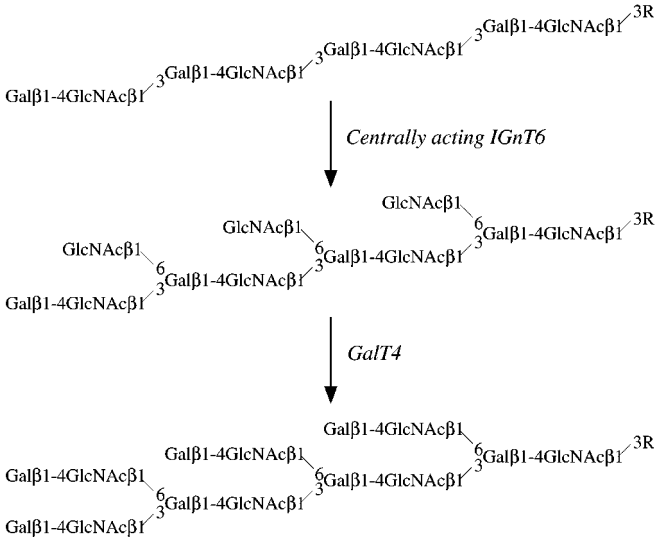


FIG. 3. **Product analysis of the branching reactions of hexasaccharide 3 catalyzed by the cIGnT6 activity of PA1 cells.** *A*, paper chromatography (solvent A for 331 h) of the products from the [^{3}H]hexasaccharide **3**. Peak *1* contained the heptasaccharide mixture (**4** + **5**) and some contaminating **3**, whereas peak *2* represented the bulk of the unchanged acceptor **3**. The position of the radiolabel in the glycans is shown by an *asterisk*. *B*, paper chromatography (solvent A for 264 h) of the products from a GalT4 reaction performed with the concentrate of [^{3}H]heptasaccharides (**4** + **5**) present in peak *1* of *A*. Peak *1* chromatographed like the authentic octasaccharide **6**, and peak 2 migrated like the hexasaccharide **3**. *C*, paper chromatography (solvent A for 115 h) of products from endo-β-galactosidase digestion of the [^{14}C]-labeled mixture of crude heptasaccharides **4** + **5**. Peak *1* chromatographed like LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3[^{14}C]Gal marker, and peak 2, like authentic tetrasaccharide **8**. The position of the [^{14}C]-label is shown by an *asterisk*. *D*, paper chromatography (solvent B for 359 h) of a partial β-*N*-acetylhexosaminidase digest of peak *2* of *C*. Peak *1* chromatographed like uncleaved **8**, peak *2* like GlcNAcβ1–6[^{14}C]Galβ1–4GlcNAc, peak 3 like GlcNAcβ1–3[^{14}C]Galβ1–4GlcNAc. *E*, paper chromatography (solvent B for 359 h) of a partial acid hydrolysate of the hexasaccharide LacNAcβ1–3(GlcNAcβ1–6)[^{3}H]Galβ1–4GlcNAcβ1–3Gal isolated from an endo-β-galactosidase digest of the crude [^{3}H]-labeled heptasaccharides **4** + **5**. Peak *1* contained a mixture of [^{3}H]-labeled trisaccharides, peak *2* was GlcNAcβ1–6[^{3}H]Galβ1–4GlcNAc, and peak *4* was GlcNAcβ1–6[^{3}H]Gal. Unlabeled markers are as in Fig. 1*C*.



SCHEME 2. **Proposed biosynthetic pathway leading to branched polylactosaminoglycan backbones in PA1 cells.** The number of branches will vary depending on the relative amounts of IGnT6, UDP-GlcNAc, and acceptor sites, but all internal galactoses of the primer chain are inherently able to react with the cIGnT6 activity. A similar pathway appears to exist in murine embryonal carcinoma cells.

branches. Such arrays may be important scaffolds for "presenting" the binding epitopes of cell adhesion saccharides in multivalent, high affinity modes. This notion is supported by the finding that functionally active sperm receptor saccharides are successfully assembled to ZP3 protein of murine zona pellucida in "appropriately" transfected murine embryonal carcinoma cells but not in a number of other cells similarly transfected (30); the failing cells probably did not express sufficient amounts of enzymes required for synthesis of branched polylactosamines. Recently, a polylactosamine backbone decorated by several sialyl Lewis X-bearing branches has actually proven to be a highly potent antagonist of lymphocyte L-selectin (31).

We note that the cDNA directing the expression of branched polylactosamines has already been isolated from the cDNA expression library from PA1 cells (32). This cDNA probably codes the cIGnT6 observed in the present experiments.

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