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Review

Enzymatic in vitro synthesis of I-branches of mammalian polylactosamines: generation of scaffolds for multiple selectin-binding saccharide determinants

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Abstract. Polylactosamines are covalent monosaccharide assemblies of the animal kingdom and some bacteria, and are characterized by backbones of interlinked *N*-acetyllactosamine units (Galβ1-4GlcNAc, LacNAc). The mammalian LacNAc arrays are linear (blood group i-type) and branched (blood group I-type), and are linked to the core elements of glycolipids as well as O- and N-glycans of glycoproteins and keratan sulfate proteoglycans. Generation of I-branches to linear i-type polylactosamines is initiated by two kinds of β6GlcNAc transferases. One type of the enzymes transfers to GlcNAcβ1-3Galβ1-OR of growing i-chains at the peridistal (underlined) Gal; these enzymes are called dIGnT (d for 'distally acting'). The other enzymes transfer to internal Gal units of preformed i-chains; they are called cIGnT (c for 'centrally acting'). Purified natural and recombinant enzymes of both types have been described. The structures of I-type polylactosamines result from a collaboration of dIGnTs, cIGnTs, β4GalTs and the i-chain-elongating iGnTs. At present, the interplay of these enzymes in vivo is poorly understood. By contrast, enzyme-assisted in vitro synthesis of branched polylactosamines representing distinct LacNAc arrays that are multiply capped by a variety of decorations is possible. Some of the synthetic polylactosamines reduce the lymphocyte-endothelium adhesion in a tissue-specific mode, raising the possibility of achieving local immunosuppression in the future. Useful applications of multiply decorated I-type polylactosamines may also be found in prevention of mammalian gamete adhesion and in inhibition of bacterial and viral adhesion to host tissues.

Key words. Polylactosamines; polylactosaminoglycans; poly-*N*-acetyllactosamines; i-antigens; I-antigens; biosynthesis; enzyme-assisted synthesis; in vitro synthesis; branched backbones; cell adhesion; selectin ligands; anti-inflammatory saccharides; endothelial saccharides; sialyl Lewis x; Le^x; sialyl diLex; sialyl diLe^x; lymphocyte extravasation; L-selectin; allograft rejection; site-specific immunosuppression; lymph nodes; inflammation; IGnT; β6GlcNAcT.

Introduction

Polylactosamines represent a group of glycoconjugates found in the animal kingdom (reviewed in [1]) and some bacteria [2], and are characterized by backbones of interlinked *N*-acetyllactosamine units (Galβ1-4GlcNAc), abbreviated here as LacNAc. This review focuses on mammalian polylactosamines.

Polylactosamines are expressed in glycolipids and in O-glycans as well as N-glycans of glycoproteins and keratan sulfate proteoglycans of mammals. Structurally, they can be considered as combinations of LacNAc

backbones and distal decorations. The backbones ride covalently anchored to the proximal lactose unit of sphingoglycolipids, to the core mannose units of N-glycans and the proximal core units of O-glycans.

Some features of the backbone structures are shown in figure 1. The primary backbones are linear assemblies of LacNAcβ1-3′LacNAc type; they represent blood group i-antigens [3]. The primary backbones often carry branches at the 6-positions of some of the galactose units; molecules of this type are known as I-branched polylactosamines because they represent blood group I-determinants. The I-branches may be short, consisting only of a GlcNAc (*N*-acetyl-D-glucosamine), a LacNAc or a decorated LacNAc unit, but they may also be quite large and structurally complex. The termini of the main chain and the branches may also carry *N*-acetyl-D-galactosamine-containing GalNAcβ1-4GlcNAc determinants that are abbreviated as LacdiNAc.

The decorations of polylactosamine backbones occur both at the primary chains and at the branches, and are exceptionally manifold; most of them are expressed only distally, but some are present all along the backbones. The decorations are often single monosaccharides such as α3- and α6-linked Neu5Ac or α2-linked Fuc on Gal, α3-, α4- or α6-bonded Fuc, or β3-linked Gal on GlcNAc, β3-, β4- or α3-linked GalNAc and α3-linked Gal on Gal; even di- or oligosaccharide groupings are expressed as caps of polylactosamine backbones. Sulfate units are also common decorations on 3- and 6-postions of Gal and the 6-position of GlcNAc. Characteristically, combinations of several terminal decorations often occur together on single backbones. A prominent example is the sialyl Lewis x determinant, Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc. The decorations expressed commonly along the polylactosamine bacbones include α3-linked Fuc units on GlcNAc and sulfate groups at position 6 of GlcNAc and Gal units.

Many of the capping elements of polylactosamines are recognized by distinct lectin proteins. Oligovalent lig-

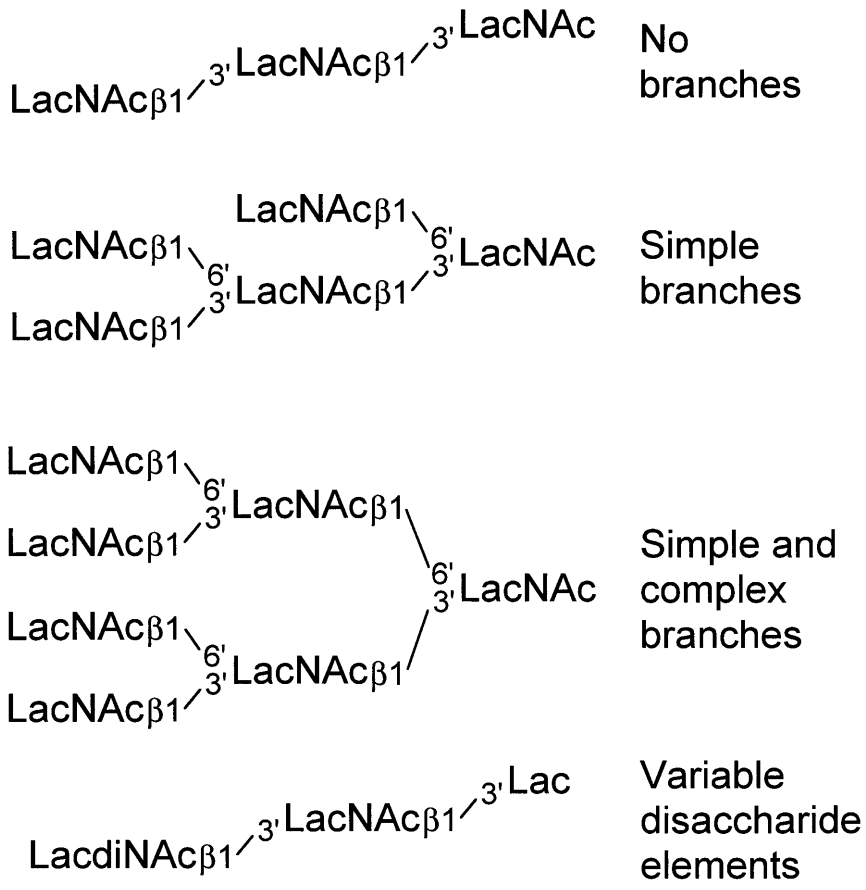


Figure 1. Structures of some archetypes of polylactosamine backbones. LacNAc, LacdiNAc and Lac represent the disaccharides Galβ1-4GlcNAc, GalNAcβ1-4GlcNAc and Galβ1-4Glc, respectively. The backbones are covalently linked at their reducing ends to glycolipids as well as to N-glycans and O-glycans of glycoproteins and keratan sulfate proteoglycans. At the distal termini the backbones carry often variable decorations consisting of saccharides and sulfate.

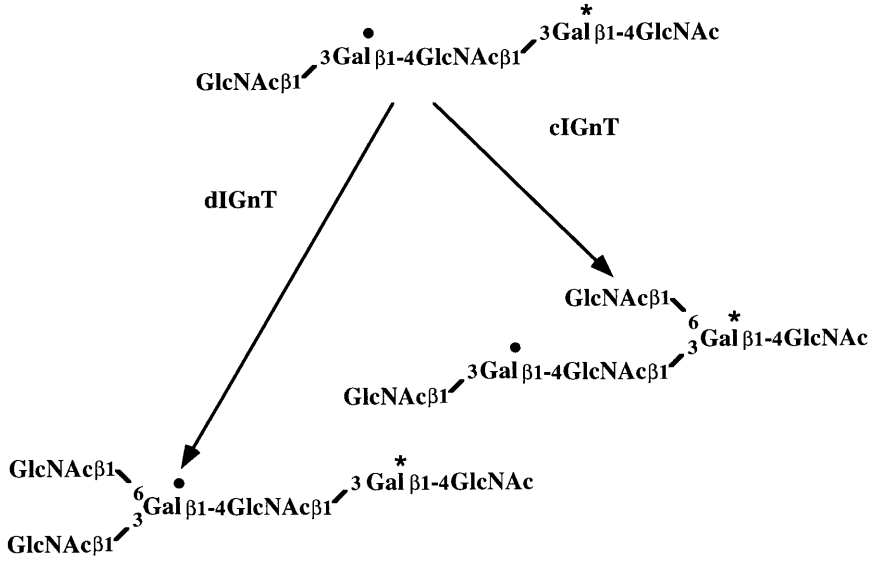


Figure 2. Two distinct enzymes catalyzing I-branching reactions of i-type polylactosamines have been observed in mammalian tissues by using the synthetic, doubly labeled pentasaccharide acceptor GlcNAcβ1-3[^{3}H]Galβ1-4GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc that possesses potential branching sites at both of the galactose units [8, 11]. The isomeric products were identified by partial random cleavage of the glycosidic linkages, ensuing fractionation of the resulting disaccharides, and identification of the radiolabel of the purified GlcNAcβ1-6Gal. In the structural formulae shown, the dot represents [^{3}H]-label and the star is [^{14}C]-label.

ands, i.e. scaffolds decorated by multiple lectin-binding determinants, are often more avidly recognized by lectins than the monovalent analogs. This makes the I-branched polylactosamines potentially important counterreceptors and antagonists of animal lectins that function as cell adhesion proteins. Notable examples include E-, L- and P-selectins, which play crucially important roles in vascular biology (reviewed in [4, 5]), and sp56, the murine sperm protein that binds to zona pellucida glycans early in the fertilization process (reviewed in [6]).

The present review aims at outlining the current understanding of enzymatic in vitro synthesis of the branches in polylactosamine backbones. There are still serious limitations in the availability of the substrates and pure enzymes, and the multivalency of the acceptors increases the experimental difficulties. Accordingly, only very preliminary data are available at present on enzyme-substrate interactions in this field. The resulting, rather naive state of our understanding is, however, amply contrasted by the putatively high practical significance of polylactosamine biology.

Two principal types of I-branching enzymes, dIGnT and cIGnT, are known

Two major types of β6GlcNAc transferase reactions that generate I-branches to polylactosamine backbones of i-type are shown in figure 2. The dIGnT reaction catalyzed by hog stomach microsomes was first studied by Piller et al. [7], and was later shown to act site specifically only at the underlined galactose residue in the pentasaccharide GlcNAcβ1-3Galβ1-4GlcNAcβ1-3′LacNAc by Helin et al. [8]. The name of the enzyme(s) responsible for this reaction is derived from the site of the action (d for 'distal'). Another type of I-branching enzyme activity was detected in mammalian serum and in human embryonal carcinoma cells in our laboratory in Helsinki, and independently in mammalian gut by Taniguchi's group in Osaka [9–13]. This β6GlcNAc transferase works at Gal units in the central positions of polylactosamine acceptors. Hence, it is often referred to as cICnT (c for 'central'). Several observations suggest that cIGnT may be more important than dIGnT in biosynthesis of branched polylactosamines in mammals.

The modus operandi of both types of I-branching β6GlcNAc transferases was established in our laboratory by identifying the primary products obtained in reactions with the doubly labeled acceptor GlcNAcβ1-3[^{3}H]Galβ1-4GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc. The branched products were treated with endo-β-galactosidase of *Bacteroides fragilis*, known to cleave the glycosidic bond of the unreacted galactose, but not that of the branch-bearing galactose [14, 15]. Paper chromatography was used to identify and quantify the cleavage products with authentic, enzymatically synthesized

oligosaccharides. Another approach involves the use of random, mild acid hydrolysis of the transferase products generated from the doubly labeled acceptor. This protocol gives mixtures of all possible di- and trisaccharides, from which the diagnostic, radiolabeled disaccharides GlcNAcβ1-6Gal can be isolated for radioisotope identification [9, 11, 16]. Matrix-assisted laser desorption/ionization mass spectral (MALDI-MS) analysis is also admirably suited to identify and quantify the cleavage products [13]. In experiments of larger scale, nuclear magnetic resonance (NMR) spectroscopy of the primary branched products has also proved to be very useful because the chemical shifts of the GlcNAc H1s of the I-branches at the reducing end LacNAc are distinct from those at midchain LacNAcs [8, 11]. Satisfactory one-dimensional (1D) ^{1}H-NMR spectra can be presently obtained with 1–2 nmol samples of medium-sized oligosaccharides.

Recently, a functional recombinant glutathionesulfotransferase (GST)-cIGnT fusion protein was described, and its substrate specificity could be established [17]. The purified recombinant enzyme of human origin catalyzed the transfer of GlcNAc from UDP-GlcNAc to the linear tetrasaccharide LacNAcβ1-3′LacNAc, converting the acceptor into the branched pentasaccharide LacNAc1-3′(GlcNAcβ1-6′)LacNAc as shown by MALDI-TOF-MS, ^{1}H-NMR spectroscopy and degradative experiments of the product. By contrast, the recombinant enzyme did not catalyze any detectable dIGnT reaction when incubated with UDP-GlcNAc and the trisaccharide GlcNAcβ1-3′LacNAc. These data are in line with the behavior of the naturally expressed cIGnTs shown in figure 2. In conclusion, the cIGnT appears to require at least one complete LacNAc-unit distal to the transfer site in functional, small acceptors. Remarkable site specificity for the galactose no. 2 (counted from the reducing end) of the pentasaccharide of figure 2 has been reported for cIGnT activities from several sources, also for a highly purified intestinal cIGnT6 [13]. However, with acceptors of larger size, the site specificity of cIGnT is sometimes not absolute. Thus, Leppänen et al. [11] reported that with the I-branched hexasaccharide acceptor GlcNAcβ1-3Galβ1-4GlcNAcβ1-3′(GlcNAcβ1-6′)LacNAc, the cIGnT activity of rat serum was able to transfer slowly even to the underlined, peridistal Gal residue. Likewise, Yeh et al. [18] have reported that a recombinant protein A-cIGnT fusion protein, which works rapidly with LacNAcβ1-3Galβ1-4GlcNAcβ1-6Manα1-6Manβ1-*O*-octyl at the underlined Gal, catalyzes slowly also the I-branching reaction at the peridistal Gal of GlcNAcβ1-3Galβ1-4GlcNAcβ1-6Manα1-6Manβ1-*O*-octyl.

Helin et al. [8], in turn observed that the primary product of the dIGnT reaction shown in figure 2 was converted into the doubly branched heptasaccharide GlcNAcβ1-3(GlcNAcβ1-6)LacNAcβ1-3(GlcNAcβ1-6′)LacNAc by continued incubation with dIGnT. Hence, dIGnT was also able to transfer to the midchain (underlined) galactose in the hexasaccharide acceptor GlcNAcβ1-3′(GlcNAcβ1-6′)LacNAcβ1-3Galβ1-4GlcNAc.

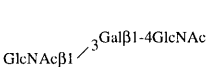
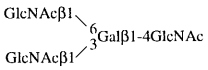
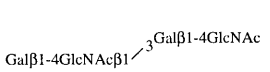
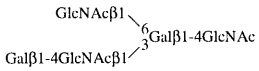
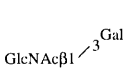
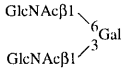
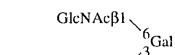
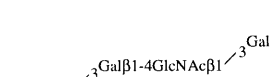
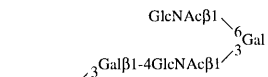
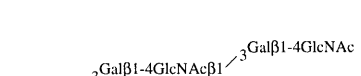
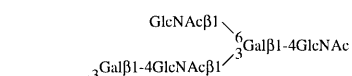
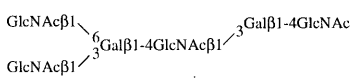
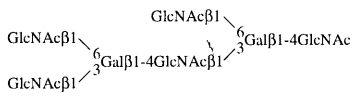
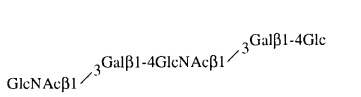
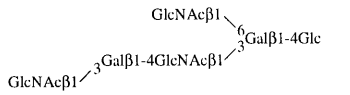
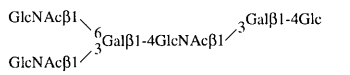
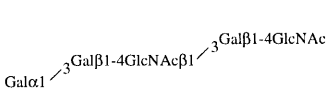
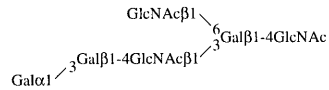
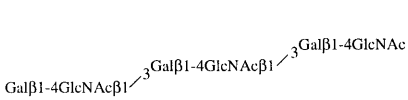
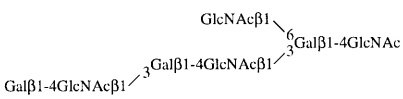
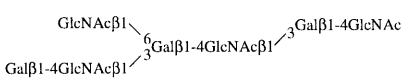
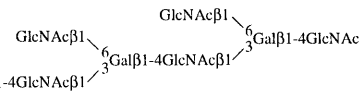
NMR spectroscopy has firmly established the structure of LacNAcβ1-3′(GlcNAcβ1-6′)LacNAc generated by the cIGnT activity of rat serum [19]

For decisive analysis of the cIGnT reaction, the ^{1}H and ^{13}C NMR spectra of both the acceptor tetrasaccharide LacNAcβ1-3′LacNAc and the pentasaccharide product (GlcNAc)(LacNAcβ1-3′LacNAc) were completely assigned [19]. Most of the proton signals were readily identified from DQFCOSY and TOCSY spectra by using as starting points the structural reporter group signals from the ordinary 1D proton spectrum. The H-5, H-6 and H-6′ resonances of the two galactoses were identified from their intraresidual rotating frame Overhauser effects (ROEs) to the distinct H-4s. After assignment of the proton signals, the corresponding ^{13}C resonances were identified in the HMQC spectra. The ^{13}C assignments in the tetrasaccharide were identical to those reported [20]. The ^{1}H and ^{13}C assignments of the pentasaccharide, in turn, were validated in the heteronuclear multiple bond correlation (HMBC) spectrum discussed below.

The structural analysis of the pentasaccharide was performed by observing the reporter group signals of the pentasaccharide, and by noting the differences in the chemical shifts of ^{1}H and ^{13}C resonances in the acceptor tetrasaccharide and in the acceptor part of the pentasaccharide product. In addition, the interresidual ROE connectivities from the newly inserted GlcNAc were analyzed, and so were the long-range interglycosidic $^3J_{C,H}$s from the heteronuclear multiple bond correlation (HMBC) spectrum of the pentasaccharide.

The proton spectrum of the pentasaccharide revealed a new doublet that was not present in the tetrasaccharide spectrum; it had the typical chemical shift (4.585 ppm) and coupling constant (8.5 Hz) of a GlcNAc residue that is β6-linked to a Gal unit [21, 22]. The H-1 and H-4 resonances of the inner Gal unit in the pentasaccharide had shifted a little to higher field, which is characteristic to I-branch-bearing Gal residues. The resonances of H-5, H-6 and H-6′ of the inner Gal residue of the tetrasaccharide experienced large changes due to the incoming GlcNAc, but the H-2 signal remained unchanged. By contrast, the proton signals of the distal Gal, the reducing-end GlcNAc as well as the inner GlcNAc were virtually identical in the tetra- and pentasaccharides. Already these data suggested strongly

Table 1. Structures of acceptor oligosaccharides and products obtained in reactions catalyzed by serum cIGnT6 activity. The relative reactivities of different acceptors during cIGnT6 reactions of rat and human serum are also shown.

Acceptor	Product(s)	Relative reactivity[a]	
		Rat serum	Human serum
 (**1**)	 (**14**)	5	2
 (**3**)	 (**15**)	200	160
 (**7**)	 (**16**)	0	not done
 (**8**)	 (**17**)	20	not done
 (**9**)	 (**18**)	12	6
 (**10**)	 (**19**)	100[b]	100
	 (**20**)	0[b]	0
	 (**21**)	c	0
 (**11**)	 (**22**)	not done	41
	 (**23**)	not done	0
 (**12**)	 (**24**)	not done	38
 (**13**)	 (**25**)	120[d]	120[e]
	 (**26**)	150[d]	140[e]
	 (**27**)	f	f

[a] Relative reactivities of the oligosaccharides were compared in parallel experiments with equimolar samples of the reference pentasaccharide **10**.
[b] The data were calculated without taking into account that **21** was formed in 3% yield.
[c] Formed in 3% yield.
[d,e] The data were calculated by taking into account that **27** carried branches at galactose nos 2 and 4 (counted from the reducing end), whereas in **25** and **26** the branches were present either at galactose no. 2 ([d]43%, [e]46%) or galactose no. 4 ([d]57%, [e]54%).
[f] The relative reactivities of the galactose nos 2 and 4 are included in the data of glycans **25** and **26**.Reproduced with permission from the PhD thesis of Anne Leppänen, 1997, University of Helsinki.

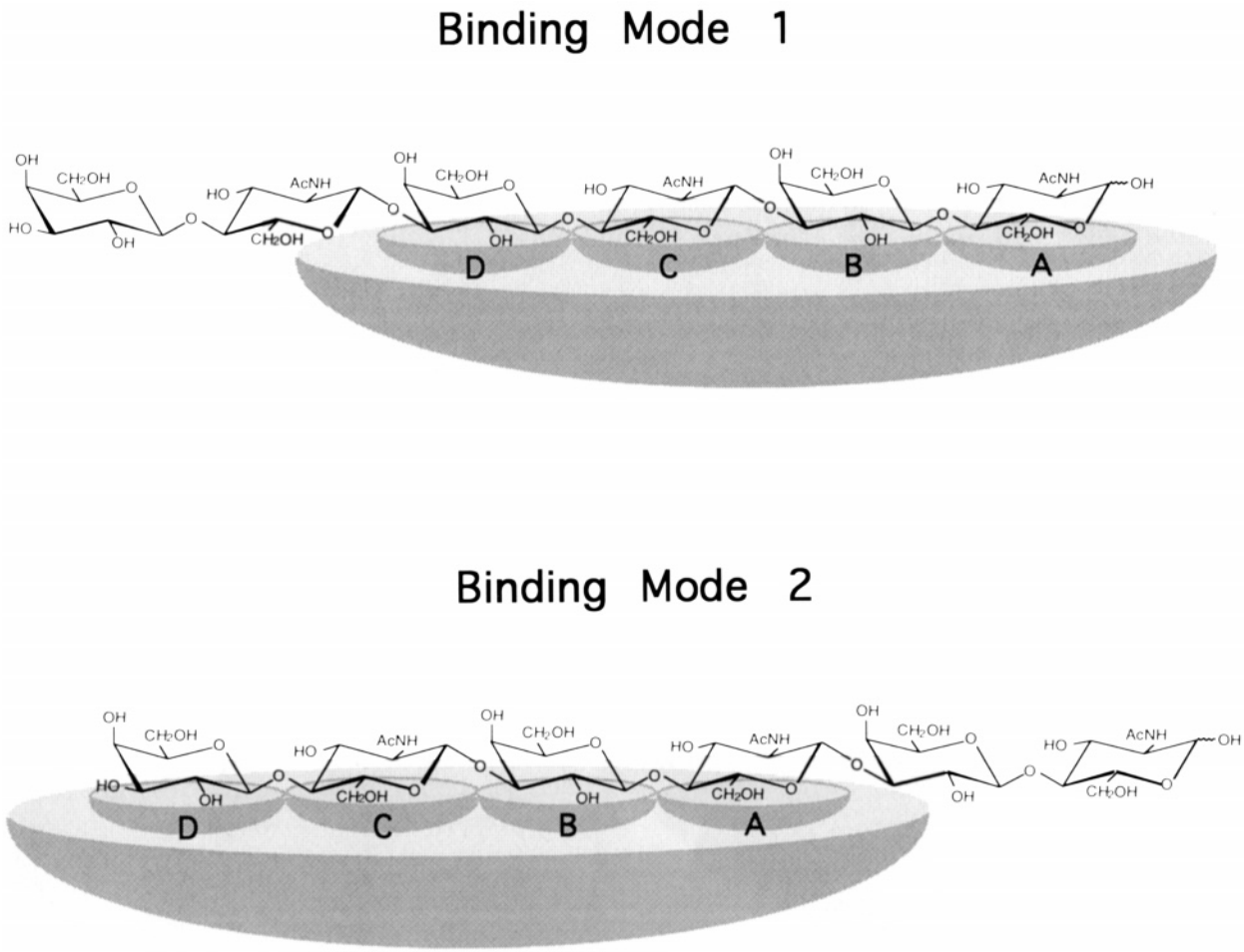


Figure 3. A model of the binding of i-type polylactosamines to cIGnTs of mammalian serum. The catalytic site of these β6GlcNAc transferases is believed to consist of four subsites (D–A), to which the tetrasaccharide determinant Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc of the acceptor is positioned for a transfer reaction to occur at the underlined Gal unit. The reactive Gal is positioned at subsite B, the adjacent upstream GlcNAc lies at subsite A, the vicinal downstream GlcNAc at subsite C and the second Gal at subsite D. The bimodal binding of the hexasaccharide acceptor LacNAcβ1-3′LacNAcβ1-3′LacNAc to the catalytic site is also shown. The binding mode 1 leads to I-branch formation at the Gal of the reducing end LacNAc, whereas the binding mode 2 yields a branch at the Gal of the middle LacNAc. Substitution at position 6 does not prevent Gal binding to subsite D, as shown by the good cIGnT reactivity at the reducing end LacNAc of the branched heptasaccharide LacNAcβ1-3′(GlcNAcβ1-6′)LacNAcβ1-3′LacNAc (see table 1). (Reproduced in modified form, with permission, from Leppänen et al., Biochemistry, 1997, 36, 7026–7036. Copyright 1997 Am. Chem. Soc.)

that the new GlcNAc in the pentasaccharide product was β1,6-bonded to the inner Gal unit.

The experiments of Maaheimo et al. [19] also revealed that only the ^{13}C resonance of C6 in the inner Gal experienced a major downfield shift due to conversion of the linear tetrasaccharide into the branched pentasaccharide. This behavior is characteristic of the carbon atom of the acceptor saccharide that resides at the site of the novel glycosidic linkage [23].

The ROE spectrum of the pentasaccharide revealed connectivities between H-1 of the newly inserted GlcNAc residue and H-6 and H-6′ of a Gal residue. Both of these Gal protons, in turn, revealed ROE contacts with H-4 of the inner Gal, confirming that this particular monosaccharide unit was close to H-1 of the new GlcNAc.

The most direct evidence for the location of the 1T6 linkage in the pentasaccharide was obtained from the HMBC spectrum, which revealed long-range couplings from the C-1 of the new GlcNAc to H-6 and H-6′ of the inner Gal, and from H-1 of the new GlcNAc to C-6 of the inner Gal. By contrast, no cross-peaks were found between the new GlcNAc and the distal Gal in the pentasaccharide.

Taken together, the NMR experiments firmly established the structure of the pentasaccharide as Lac-

NAcβ1-3′(GlcNAcβ1-6′)LacAc. The 2D-NMR experiments were performed using a 500-MHz NMR spectrometer, and 1.8 μmol of the tetrasaccharide and 2.7 μmol of the pentasaccharide. Recently, much smaller tetra-, penta- and hexasaccharide samples, amounting to only about 0.5 μmol, have given satisfactory HMBC spectra in the nanoprobe of our laboratory.

Relative reactivities of small i-type acceptors in cIGnT reactions suggest a tentative model of acceptor-enzyme recognition [11, 17]

Comparative I-branching experiments of Leppänen et al., involving a panel of equimolar samples of several radiolabeled acceptors of polylactosamine type and the cIGnT activity of human blood serum, revealed that the tetrasaccharide LacNAcβ1-3′LacNAc is the smallest acceptor showing full reactivity (Table 1) [1, 9, 11]. This was true also for the cIGnT activity of rat serum.

The data revealed important contributions to the reactivity by the upstream GlcNAc- and Glc neighbors of the accepting Gal residue; LacNAcβ1-3′Lac(NAc) reacted faster than LacNAcβ1-3Gal. (Note, the terms upstream and downstream refer to the direction towards the reducing end of the chain, and towards the nonreducing end of the chain, respectively.) However, additional growth of the acceptor in the upstream direction did not further the reactivity with cIGnT; the reactivity of the hexasaccharide LacNAcβ1-3′LacNAcβ1-3′LacNAc at the underlined LacNAc was not improved in comparison with the tetrasaccharide LacNAcβ1-3′LacNAc (see table 1). Even the downstream GlcNAc neighbor of the reacting galactose appeared to improve the cIGnT reaction; the serum enzyme did not transfer to the Gal of distal, unsubstituted LacNAc but worked slowly with GlcNAcβ1-3′ → LacNAc. As discussed above, a large, characteristic increment in the cIGnT reactivity was associated with further downstream elongation of the trisaccharide to the tetrasaccharide acceptor LacNAcβ1-3′LacNAc. However, further downstream elongation of the acceptor chain by an additional β1,3′-linked GlcNAc, or by a complete β1,3-linked LacNAc unit, did not improve the reactivity.

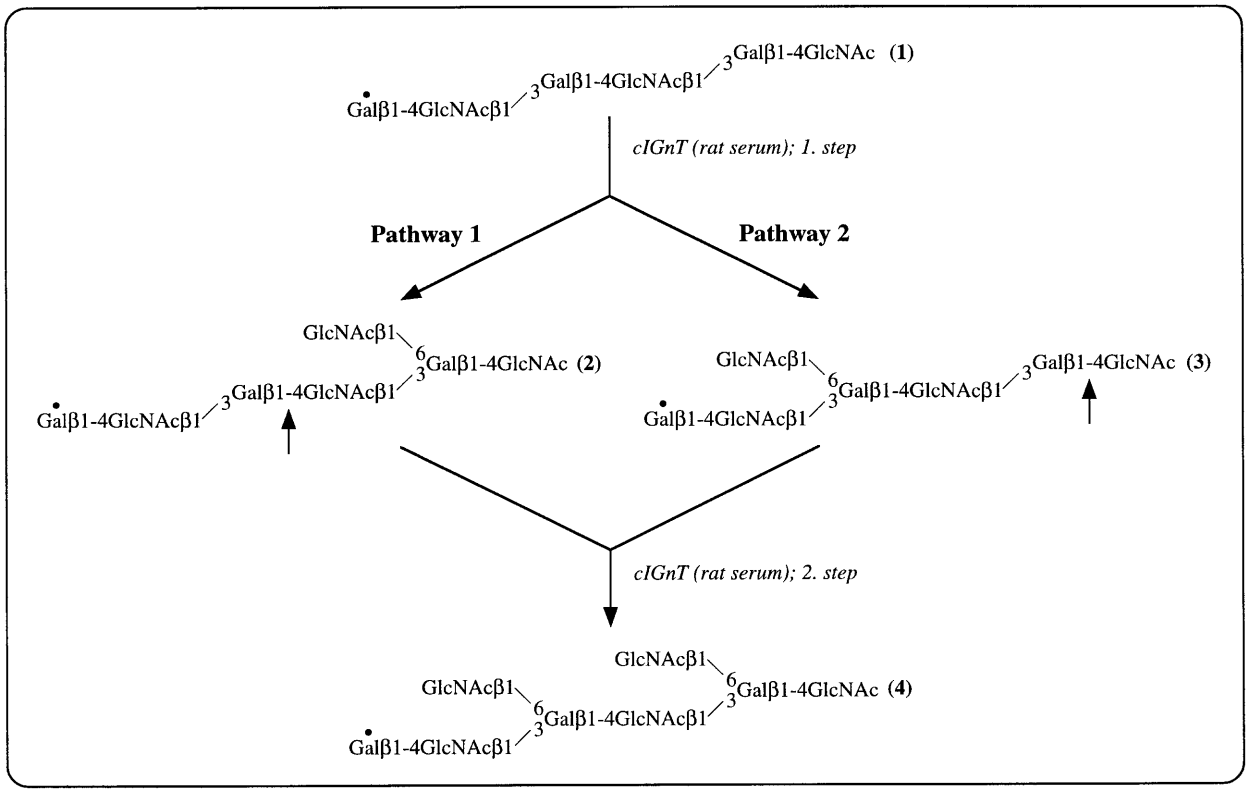


Figure 4. Two in vitro pathways catalyzed by cIGnT activity of rat serum, leading from the linear hexasaccharide LacNAcβ1-3′LacNAcβ1-3LacNAc to the doubly branched octasaccharide LacNAcβ1-3′(GlcNAcβ1-6′)LacNAcβ1-3′(GlcNAcβ1-6′)LacNAc. The dot shows the position of radiolabel that was useful in quantifying the intermediary heptasaccharide isomers by endo-β-galactosidase digestion (the upright arrows show sites of endo-β-galactosidase action) and ensuing radiochromatography. (Reproduced in modified form, with permission, from Leppänen et al., Biochemistry 1997, 36, 7026–7036. Copyright 1997 Am. Chem. Soc.)

These early data suggested a tentative model of the optimal binding between cIGnT and the i-chain acceptors [11]. The model shown in figure 3 involves (i) four distinct subsites (A, B, C and D) on the enzyme surface, and (ii) the linear, four-monosaccharide-determinant Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc in the acceptor. The transferase reaction takes place at the underlined Gal unit at the subsite B.

The complete set of experiments supporting this model has not yet been repeated with the pure recombinant cIGnT from PA1 cells that has recently become available in two laboratories [17, 18, 24]. But the trisaccharide GlcNAcβ1-3Galβ1-4GlcNAc sequence was shown to react poorly, whereas the tetrasaccharide Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc determinant reacted efficiently with the purified recombinant enzymes of both laboratories. This implies that at least both Gals of the tetrasaccharide sequence are also crucial for reactions catalyzed by the purified recombinant cIGnTs.

cIGnT reactions generate branches at adjacent LacNAc units of i-type chains [11]

Leppänen et al. [11] performed important in vitro I-branching reactions also with the linear radiolabeled hexasaccharide LacNAcβ1-3′LacNAcβ1-3′LacNAc. The reactions, catalyzed by the cIGnT activity of rat serum, proceeded stepwise leading first to an intermediary mixture of two isomeric, branched heptasaccharides as outlined in figure 4. Here, the β6GlcNAc transfer to Gal 4 of the acceptor (i.e. to the monosaccharide no. 4, counted from the reducing end at right) proceeded slightly faster than the transfer to Gal 2, but the rate difference was quite small. For analysis, the mixture of the [^{3}H]heptasaccharide intermediates was digested exhaustively with endo-β-galactosidase. The digest contained two radiolabeled products, namely the hexasaccharide [^{3}H]LacNAcβ1-3′(GlcNAcβ1-6′)LacNAcβ1-3Gal and the trisaccharide [^{3}H]LacNAcβ1-3Gal, which were separated and identified by paper chromatography. They originated from the isomeric heptasaccharides [^{3}H]LacNAcβ1-3′(GlcNAcβ1-6′)LacNAcb1-3′LacNAc and [^{3}H]LacNAcβ1-3′LacNAcb1-3′(GlcNAcβ1-6′)LacNAc, respectively, and contained nearly equal amounts of [^{3}H]Gal radioactivity. These data suggest that the initial interaction of the linear hexasaccharide [^{3}H]LacNAcβ1-3′LacNAcβ1-3′LacNAc and cIGnT of rat serum involves two distinct modes of functional binding that are shown in figure 3. Experiments performed with sera from other mammalian species gave similar results.

Analogous experiments were recently performed also with the glycolipid acceptor LacNAcβ1-3′LacNAcβ1-3′LacNAcβ1-6Manα1-6Manβ1-*O*-octyl and a purified recombinant protein A-cIGnT fusion protein by Ujita et al. [24]. Even these incubations resulted in I-branching at both internal LacNAc units, but here, the LacNAc adjacent to the mannobiose unit reacted more slowly than the peridistal LacNAc.

In the second stage of the I-branching reaction of figure 4, both intermediary products seemed to be functional acceptors. A preparative scale experiment involving a sample of 438 nmol of the hexasaccharide acceptor yielded 254 nmol of the doubly branched octasaccharide in purified form and 78 nmol of a mixture of the intermediary, singly branched heptasaccharides [11]. If only one of the intermediary products had reacted further, the presence of roughly equal amounts of the octasaccaharide and the heptasaccharide fractions would have been expected at the end of the reaction. This view was confirmed by experiments performed with pure isomers of the heptasaccharide intermediates. The data implied that the preformed branches did not prevent the second I-branching reaction at the adjacent upstream LacNAc nor at the vicinal downstream LacNAc [11].

Leppänen et al. [12] have generated the doubly branched octasaccharide of figure 4 from the mixture of the two singly branched, isomeric heptasaccharide intermediates even in a reaction catalyzed by the prototype cIGnT of human embryonal carcinoma cells of line PA1. Ujita et al. [24] have also obtained doubly branched polylactosamine backbones of the type of LacNAcβ1-3′(GlcNAcβ1-6′)LacNAcβ1-3′(GlcNAcβ1-6′LacNAcb1-OR in a reaction catalyzed by the purified recombinant hybrid protein consisting of protein A and the cIGnT from the PA1 cells.

The purified recombinant GST-cIGnT fusion protein described by Mattila et al. [17] catalyzed in a partial reaction the transfer of one and two new GlcNAc residues to the octasaccharide acceptor LacNAcβ1-3′LacNAcβ1-3′LacNAcβ1-3′LacNAc. The missing third branch was not found, probably because the small amount of the enzyme available severely restricted the overall extent of the reaction. It is likely that the enzyme binds the acceptor functionally in several distinct ways that resemble those of figure 3. This view is supported by the data reported by Salminen et al. [25], who transferred β1,6-bonded GlcNAc branches to all internal galactoses of the octasaccharide LacNAcβ1-3′LacNAcβ1-3′LacNAcβ1-3′LacNAc by using the cIGnT activity of rat serum.

In conclusion, all internal LacNAc units of unsubstituted i-chains are inherently able to become I-branched in reactions catalyzed by cIGnTs originating from mammalian blood serum or from human embryonal carcinoma cells of line PA1. A preformed GlcNAc branch at the 6′ position of an internal LacNAc unit does not prevent branch formation at internal LacNAc

units of adjacent downstream or upstream positions. The flexible β1,6 linkages, joining the I-branches to the i-chains, appear to eliminate any potential steric problems in the acceptor binding.

In preferring extended i-type polylactosamine determinants as acceptors, cIGnTs resemble human milk α3-fucosyltransferases, which require a complete LacNAc unit as their acceptor and are apparently unable to act with the monosaccharide form of the acceptor site. Moreover, they react much better with the trisaccharide GlcNAcβ1-3′LacNAc than with the disaccharide LacNAc [26]. Extended areas of the i-type chains are probably involved also in other *endo*-type reactions of polylactosamine metabolism. In this respect, polylactosamine biochemistry bears some resemblance to classical polysaccharide degradation, e.g. the action of cellulases, lysozymes and endochitinases on their substrates.

cIGnT reactions of α3-fucosylated polylactosamine chains [27]

The cIGnT activity of rat serum was unable to work with the fucose-containing pentasaccharide LacNAcβ1-3Galβ1-4(Fucα1-3)GlcNAc (abbreviated here as LacNAcβ1-3′Lex), although the I-branch was readily transferred to the fucose-free tetrasaccharide LacNAcβ1–3′LacNAc [27]. Even the purified, functional recombinant GST-cIGnT fusion protein was unable to generate the I-branch to LacNAcβ1-3′Lex [17]. The data imply that the Lex determinant cannot replace the fucose-free LacNAc at the reactive site of functional acceptors of cIGnT. This is perhaps not very surprising, because in aqueous solutions the galactose and the fucose of the Lex determinant are tightly associated—in fact, stacked on top of each other [28]—and many proteins recognizing readily unsubstituted LacNAc units are unable to bind Lex. Pertinent examples include exo-β-galactosidases (reviewed in [29]), endo-β-galactosidases of *Bacteroides fragilis* and *Escherichia freundii* (reviewed in [1]), iGnT [30], α3-sialyltransferase [31], wheat germ agglutinin [32, 33] and *Datura stramonium* agglutinin [34].

The cIGnT activity of rat serum was also unable to generate branches to the fucose-containing pentasaccharide Lexβ1-3′LacNAc [27]. The ability of a Lex group to inhibit the branching at the adjacent upstream LacNAc unit is probably also caused by the peculiar stacking of the fucose and the distal galactose, which obviously prevents the appropriate binding of the Lex determinant to the putative subsites C and D on the surface of cIGnT (see fig. 3).

In line with the above data, the cIGnT activity of rat serum was unable to generate branches to the fucose-containing heptasaccharide LacNAcβ1-3′Lexβ1-3′LacNAc [27]. In the isomeric heptasaccharide LacNAcβ1-3′LacNAcβ1-3′Lex, the inhibition of the branch-forming reaction was restricted to the fucose-carrying Lex unit; at the middle LacNAc of the heptasaccharide, the I-branching proceeded normally [27]. However, in the third isomeric heptasaccharide Lexβ1-3′LacNAcβ1-3′LacNAc, the fucose residue of the distal Lex prevented the cIGnT reaction completely at the middle LacNAc and almost completely even at the reducing end LacNAc [27]. The apparent long-distance inhibition caused by the distal Lex determinant may in fact be mediated via disturbed binding of the adjacent Gal of the middle LacNAc at the putative subsite D of cIGnT. We are not aware of any previous reports suggesting distinct intramolecular interactions between a Lewis x group and the adjacent upstream galactose of a polylactosamine chain. Hence, the distal Lex epitope may be inhibitory because it causes steric problems to the binding between cIGnT and the middle LacNAc of the acceptor.

The inhibitory effects exerted by the Lex groups on cIGnT action have served us well in protecting the heptasaccharide LNβ1-3′Lexβ1-3′LN from untimely I-branching catalyzed by serum cIGnT during an elongation reaction with serum iGnT [25]. The resulting GlcNAcβ1-3′LNβ1-3′Lexβ1-3′LN was subsequently defucosylated by mild acid hydrolysis [35], and the i-type heptasaccharide product was purified and β4-galactosylated to afford the linear octasaccharide backbone LacNAcβ1-3′LacNcβ1-3′LacNAcβ1-3′LacNAc.

Is site-specific I-branching possible via site specific α3-fucosylation of polylactosamines?

The site-specific inhibition of cIGnT reactions discussed above prompts us to ask whether appropriately fucosylated polylactosamines can be synthesized and used to generate site-specifically branched I-type glycans. This is an attractive possibility because optimally spaced branches carrying appropriate, clustery decorations on flexible polylactosamine scaffolds afford high avidity binding to cell surface lectins (see below). In addition, the fucose can be removed afterwards by mild acid hydrolysis under conditions that do not cleave the glycosidic linkages of the backbone [35]. The experiments discussed above already represent a beginning, allowing the synthesis of LacNAcβ1-3′(GlcNAcβ1-6′)LacNAcβ1-3′LacNAc and LacNAcβ1-3′LacNAcβ1-3′(GlcNAcβ1-6′)LacNAc in pure form.

Further experiments have enabled us to synthesize several kinds of i-type polylactosamines that are site-specifically α3-fucosylated at the reducing end terminus. One method is based on use of affinity chromatography

on immobilized wheat germ agglutinin that directly separates Rβ1-3′LacNAcβ1-3′Lex from all other isomers carrying the single α3-bonded fucose in different positions along the i-chain [33]. Another method of synthesis is based on α3-fucosylation of GlcNAcβ1-3′LacNAc before further elongation and modification of the chain [36, 37]. By combining these two principles, we have been able to synthesize several i-type chains that contain the α3-fucose in the second LacNAc unit (counted from the reducing end). A simplified outline of the process actually used in the experiments of Niemelä et al. [33] is as follows: the pentasaccharide GlcNAcβ1-3′LacNAcβ1-3′LacNAc was partially fucosylated by restricting the amount of the donor and/or the reaction time. The mixture of monofucosylated products was then separated from the difucosylglycans and from the original acceptor by chromatography. The two monofucosylated products, viz. GlcNAcβ1-3′Lexβ1-3′LacNAc and GlcNAcβ1-3′LacNAcβ1-3′Lex, were separated by affinity chromatography on immobilized wheat germ agglutinin. The pure isomers could then be elongated by successive β4GalTI and iGnT reactions. A synthesis of this type would yield pure LacNAcβ1-3′LacNAcβ1-3′Lexβ1-3′LacNAc, for instance. The postulated product of a cIGnT reaction with this acceptor, in turn, would be LacNAcβ1-3′(GlcNAcβ1-6′)LacNAcβ1-3′Lexβ1-3′LacNAc, because neither the Lex group nor the reducing end LacNAc would react. Fucose removal by mild acid hydrolysis would convert this intermediate into the site-specifically I-branched backbone LacNAc-β1 - 3′(GlcNAcβ1 - 6′)LacNAcβ1 - 3′LacNAcβ1 - 3′LacNAc.

It is important that the i-type polylactosamines can be α3-fucosylated selectively at the distal end of the chains by using human recombinant Fuc TIX [38]. Alternatively, human recombinant Fuc TVII may be used to fucosylate α3-sialylated polylactosamines site specifically at the distal, sialylated LacNAc unit [39, 40]. Subsequently, the fucosylated intermediate may be enzymatically desialylated if needed, but the sialyl Lewis x group probably exerts a branch-directing influence similar to that of the unsialylated Lewis x.

α3-Fucosylation restricted to the distal LacNAc can also be achieved by transferring temporary β6-bonded GlcNAc branches at all internal Gal units of the i-chains. They will protect all internal LacNAc units, making them unreactive with Fuc TIV [40] and with the α3/4 FucTs of human milk [26]. The protected acceptors can then be fucosylated selectively at the distal LacNAc unit. Finally, the β6-linked GlcNAc branches can be removed from the intermediates by a treatment with an appropriate β-*N*-acetylglucosaminidase, affording the distally fucosylated i-chains [41].

Random monofucosylation at all positions of the i-chain and subsequent isolation of the distally fucosylated isomer is also possible. This has been achieved by removing all other isomers by a treatment with a mixture of exo-β-galactosidase and exo-β-*N*-acetylglucosaminidase followed by chromatography [33]. The exohydrolases are unable to cleave the Lex group at the downstream terminus, but they effectively remove the terminal LacNAc residues that are not protected by the presence of the α3-bonded fucose. The data of Leppänen et al. [27] suggest that the distally fucosylated chain Lexβ1-3′LacNAcβ1-3′LacNAcβ1-3′LacNAc, for instance, should be branched effectively only at the proximal LacNAc unit.

In conclusion, site-specific α3-fucosylation of i-type polylactosamines is already relatively well developed, furnishing chains that carry the fucosyl unit solely at the first, the second or the last LacNAc unit, respectively. The site specifically fucosylated chains, in turn, react site specifically with cIGnT, restricting branch generation to a few, selected midchain positions. Removal of the temporary fucosyl units from the branched intermediates by mild acid treatment furnishes the site-specifically branched I-glycans.

Naturally, the dIGnT reaction is quite useful for selective branch generation at the distal end of the i-chains.

Some dIGnTs transfer in vitro to several types of O- and N-glycans as well as to glycolipid glycans

A mucin β6*N*-acetylglucosaminyltransferase was purified to apparent homogeneity from Golgi membranes of bovine tracheal epithelium, and was shown to convert GlcNAcβ1-3Galβ1-OMe into GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-OMe [42]. Hence, the enzyme can be regarded as a highly purified sample of dIGnT. Somewhat surprisingly, this enzyme also used core 1 (Galβ1-3GalNAcα1-OR) and core 3 (GlcNAcβ1-3GalNAcα1-OR) mucin oligosaccharides as acceptor substrates, converting them to core 2 [Galβ1-3(GlcNAcβ1-6)GalNAcα1-OR] and core 4 [GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα1-OR] structures, respectively. Accordingly, the transferase can be classified as core 2 β6GnT (C2GnT) and/or core 4 β6GnT (C4GnT) equally well as dIGnT. A relaxed substrate specificity has also been observed with the crude amalogous enzymes from pig gastric mucosa [43, 44], Novikoff ascites tumor cells [21] and human ovarian tissue [45].

The crude dIGnT activity from pig gastric mucosa also transfers effectively to the penultimate Gal of the GalNAcβ1-3Galα1-OR sequence in globo-*N*-tetraose, generating the branched pentasaccharide GalNAcβ1-3(GlcNAcβ1-6)Galα1-4Galβ1-4Glc [46]. Although globoside is a common glycolipid in animal tissues, the branched glycolipid GalNAcβ1-3(GlcNAcβ1-6)Galα1-

4Galβ1-4Glcβ1-ceramide has not been reported. This is a little surprising, because the gastric mucosal enzyme is capable of transferring a β6-linked GlcNAc efficiently to free as well as lipid-bound trisaccharide GlcNAcβ1-3Galβ1-4Glc in vitro [7, 21]. The apparent nonaccessibility of globoside in cell membranes to dIGnT, as well as to galactose oxidase [47] and to antibodies [48], may have a common cause that remains unidentified at present. A highly interesting possibility is that homo- or heterotypic saccharide-saccharide interactions involving globoside molecules (and possibly other glycolipids) of a given membrane may lead to clustered structures that are not accessible to dIGnT of the same membrane. Clusters of this type may well be present in the putative glycosphingolipid-enriched domains of cell membranes [49].

As the crude dIGnT from pig gastric scrapings transfers to GalNAcβ1-3Galα1-OR (globotetraose) as well as to the Galβ1-3GalNAcα1-OR (core 1), it is possible that it will transfer a β6-linked GlcNAc also to Galβ1-3Galα1-OR and GalNAcβ1-3GalNAcα1-OR determinants at the underlined monosaccharide units. Recently, this promiscuous enzyme was cloned, functionally expressed and purified by Yeh et al. [18] as well as by Schwientek et al. [50]. The purified recombinant enzyme used analogs of core 1 and core 3 mucin oligosaccharides as acceptors quite effectively in both laboratories, and appeared to work also with polylactosamine acceptors in the experiments of Yeh et al. [18].

dIGnT is also able to join forces with iGnT for in vitro modification of N-glycans. All distal Gal residues of bi-, tri- and tetraantennary asialo N-glycans have been converted step by step into Galα1-3′LacNAcβ1-3(Galα1-3′LacNAcβ1-6)Gal determinants in vitro [51]. In this way tetra-, hexa- and octavalent Galα1-3′LacNAc glycans were generated. They resemble glycan **B** of figure 6, and may well inhibit murine gamete adhesion efficiently (see below). The identity of the final products was ascertained by one-dimensional (1D) NMR experiments and MALDI-TOF-MS. It is remarkable that these in vitro reactions yielded products in which the elongation and branching were completed at all branches of the original N-glycan acceptors. The naturally occurring polylactosamines in N-glycans are not randomly distributed among the different antennae, but are mostly present in nonerythrocyte sources in the 6-branch of the 2,6-branched mannose [52].

In vitro β-galactosylation of the GlcNAc branches

Partial β4-galactosylations of the branched tetrasaccharide GlcNAcβ1-3′(GlcNAcβ1-6′)Lac with the β4-GalTI of bovine milk revealed that at the 6-linked arm of the acceptor reacted much faster than the 3-linked branch [53]. At an early stage of the reaction, when only 6% of the available acceptor sites had reacted, the products represented a mixture of LacNAcβ1-6′(GlcNAcβ1-3′)Lac (97%) and LacNAcβ1-3′(GlcNAc-β1-6′)Lac (3%). At a later stage, when 85% of the available acceptor sites were filled, the diminishing pentasaccharide fraction consisted solely of LacNAcβ1-6′(GlcNAcβ1-3)′Lac. Hence, among the two pentasaccharides formed in the initial stages of the reaction, the one with galactose-less 6-arm reacted fastest at the second step of the galactosylation.

Analogous data were obtained in a set of partial β4-galactosylation experiments with the branched tetrasaccharide GlcNAcβ1-3′(GlcNAcβ1-6′)LacNAc and β4GalTI [53]. At the early stages of the reaction, the pentasaccharide LacNAcβ1-6′(GlcNAcβ1-3′)LacNAc was formed almost three times more rapidly than the isomeric LacNAcβ1-3′(GlcNAcβ1-6′)LacNAc. During the second stage of the reaction, the pentasaccharide LacNAcβ1-3′(GlcNAcβ1-6′)LacNAc was consumed faster than the isomeric LacNAcβ1-6′(GlcNAcβ1-3′)LacNAc. Hence, the 6-linked GlcNAc arm was consistently more reactive than the 3-linked GlcNAc arm. The data of Maaheimo et al. [54] suggest that the high β4GalTI-reactivity of the 6-linked GlcNAc-branch in the distal GlcNAcβ1-3(GlcNAcβ1-6)LacNAc determinant may be a general property of this epitope in several different types of polylactosamines.

The β4GalTI may be the most important GalT in elongating the i-type chains of polylactosamines [24]. Hence, it may be significant that the pyridylaminylated trisaccharide GlcNAcβ1-3Galβ1-4Glc-PA reacts faster than the 6-linked GlcNAc of the I-branched pentasaccharide LacNAcβ1-3(GlcNAcβ1-6)Galβ1-4Glc-PA with β4GalTI at small acceptor concentrations [24]. The difference in the K_M values of the two acceptors was eightfold. In view of these data the authors suggest that a concerted action of cIGnT and β4GalTI leads preferentially to long i-chains with short I-branches. Such LacNAc arrays have been identified for instance in erythrocytes [55–57] and in human embryonal carcinoma cells of line PA1 [58].

Further elongation of LacNAc-branches by iGnT reactions

Polylactosamines are also known that carry large and complex branches of more than one LacNAc unit [59] (see fig. 1). Biosynthesis of the large branches requires that additional β1,3GlcNAc transferase reactions (iGnT reactions) initiate the growth of the small I-branches consisting of a single LacNAc unit.

The relative rates of iGnT-elongation reactions at β1,6-linked and β1,3-linked LN arms of polylactosamines

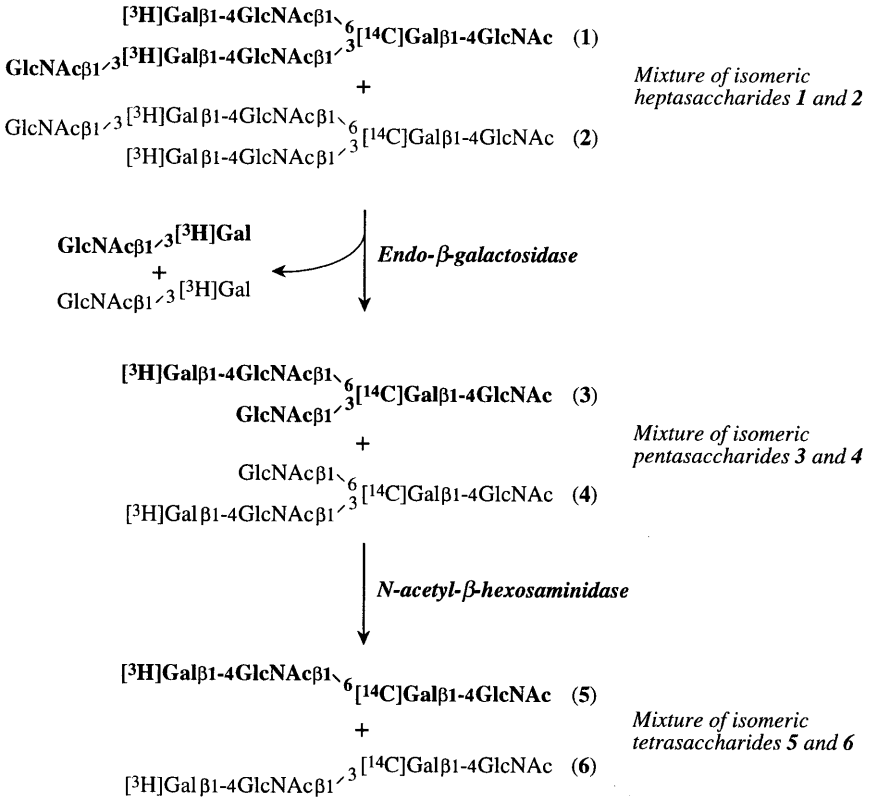


Figure 5. Outline of the analysis of the mixture of the isomeric [^{3}H]heptasaccharides **1** and **2**, generated in an iGnT reaction of the branched hexasaccharide LacNAcβ1-3′(LacNAcβ1-6′)LacNAc**.** The analysis consisted of cleavage reactions that removed the elongated branches from both heptasaccharides. Two types of fonts are used to show that the tetrasaccharide **5** was derived from the heptasaccharide **1**, whereas **6** was derived from **2**. The isomeric tetrasaccharides **5** and **6** could be separated by chromatography, whereas the heptasaccharides **1** and **2** are not separable by our present methods. (Reproduced in modified form, with permission, from Vilkman et al., Carbohydrate Research, 1992, 226, 155–174. Copyright 1992, Elsevier Scientific Publishers.

were compared in a series of in vitro experiments reported by Vilkman et al. [60]. These early reactions were performed with the iGnT activity present in human serum. Incubation of radiolabeled hexasaccharide LacNAcβ1-3′(LacNAcβ1-6′)LacNAc with UDP-GlcNAc and serum gave initially a transient 1:1 mixture of two isomeric heptasaccharides, GlcNAcβ1-3′LacNAcβ1-3′(LacNAcβ1-6′)LacNAc and LacNAcβ1-3′(GlcNAc-β1-3′LacNAcβ1-6′)LacNAc. The two heptasaccharides reacted further in the incubation mixture to form the radiolabeled octasaccharide GlcNAcβ1-3′LacNAcβ1-3′(GlcNAcβ1-3′LacNAcβ1-6′)LacNAc.

The heptasaccharide intermediates could not be separated from each other chromatographically, but they could be detected, identified and quantified by stepwise enzymatic degradations that gave characteristic, separable products. The radiolabeled heptasaccharide mixture was treated first with endo-β-galactosidase of *B. fragilis* and then with β-*N*-acetylhexosaminidase as shown in figure 5. The endo-β-galactosidase reaction generated two isomeric pentasaccharides, which were converted into a mixture of two isomeric, linear tetrasaccharides in the ensuing β-*N*-acetylhexosaminidase reaction. The tetrasaccharides, in turn, could be separated reliably from each other and quantified by chromatography as described [61]. The data revealed that in the initial phases of the iGnT reaction, the two branches of the hexasaccharide LacNAcβ1-3(LacNAcβ1-6)LacNAc had reacted equally well, generating a nearly equimolar mixture of the heptasaccharides **1** and **2** of figure 5. The composition of the mixture of the heptasaccharide intermediates remained unchanged even at the later stages of the reaction, i.e. during the conversion of the heptasaccharides into the octasaccharide. This showed that even the two intermediary heptasaccharides reacted equally well with the iGnT.

Analogous experiments were reported also for the biantennery pentasaccharide LacNAcβ1-3(LacNAcβ1-6)Gal [60]. Even this acceptor reacted equally well at both of its antennae with the serum iGnT. However, the

recent experiments of Ujita et al. [24] involving a recombinant, purified protein A-iGnT fusion protein, UDP-GlcNAc and pyridylaminylated lacto-*N*-neohexaose, LacNAcβ1-3(LacNAcβ1-6)Galβ1-4Glc-PA, and an analysis scheme identical to that of figure 5, revealed a much higher reactivity in the 6-linked branch of the acceptor than in the 3-linked branch.

Accordingly, in tissues possessing significant dIGnT activity in comparison to β4GalTs, the formation of the distal GlcNAcβ1-3(GlcNAcβ1-6′)LacNAcβ1-OR determinant, combined with the ensuing, fast β4-galactosylations (see above) and efficient iGnT reactions at the 6-arm of the intermediary epitopes, will probably lead to generation of large and complex backbone branches. Indeed, quite large and complex branch structures are expressed among the sphingoglycolipids of hog gastric mucosa, at the site of a particularly high dIGnT activity [59].

An integrated view of the branch biosynthesis is still missing

A clear concept of the biosynthesis of branched variants among polylactosamine backbones is difficult to construct on the basis of presently available kinetic data. Obviously, much further information is required before a true understanding of the biosynthetic growth of of the different types of branched polylactosamines is possible.

The activities and substrate specificities of collaborating dIGnT, cIGnT, β4GalTs and iGnTs will determine in part the structures of the resulting I-type polylactosamine in different tissues. For instance, it may be important that dIGnT and β4GalTs compete for the same substrates. This implies that multiple distal branching may be possible only in cells expressing dIGnT activity at levels that are comparable to those of the β4GalTs.

Also, the spatial localization of the transferases in relation to each other in the Golgi membranes is of decisive importance for the final outcome of the branch biosynthesis in a given type of cells. For instance, the length of the branches will be determined by the activities and by the locatization of cIGnT and iGnT in the Golgi membranes; if the bulk of iGnT is centrally located compared to cIGnT, the I-branches will be predominantly short.

Some of the present confusion about the branch biosynthesis is probably caused by mammalian expression of several distinct β4GalTs [62, 63], at least two iGnTs [64, 65], and possibly also several dIGnTs and cIGnTs. Finally, the conjugated forms of polylactosamines, e.g. those present at the different glycosylation sites of indi-

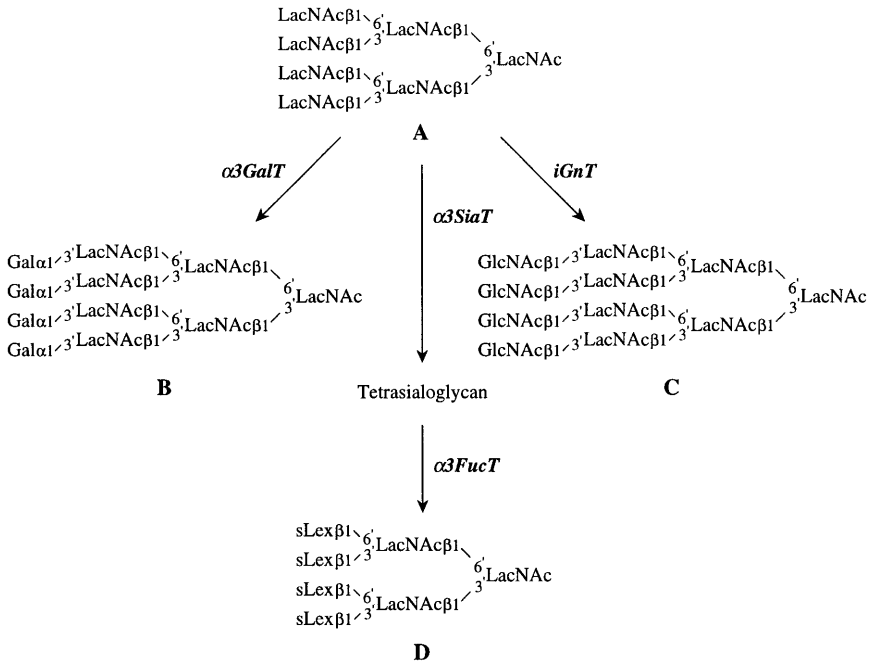


Figure 6. Examples of enzymatically synthesized, highly branched polylactosamines that carry several distal decorations. Glycan **B** is a potent inhibitor of murine gamete adhesion, and Glycan **D** is a very potent antagonist of lymphocyte L-selectin, which implies that it may possess important antiinflammatory properties. sLex represents the tetrasaccharide determinant Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc.

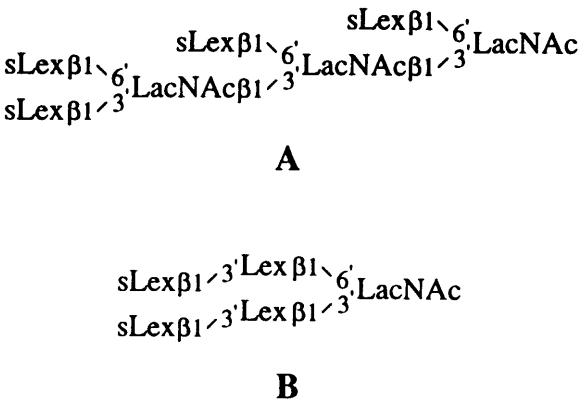


Figure 7. Structures of two heavily decorated I-type polylactosamines of interesting biological properties. Glycan **A** is an equally potent antagonist of lymphocyte L-selectin as Glycan **D** of figure 6. Glycan **B** of figure 7, in turn, was shown to prevent L-selectin-mediated lymphocyte-endothelium adhesion specifically in inflamed tissue, it was much less efficient in lymph nodes.

vidual glycoproteins, will probably have acceptor properties that are different from the free glycans.

In vitro synthesis of branched polylactosamines representing potent inhibitors of lectin-mediated cell adhesion

In clear contrast to the slow advances in true understanding of branch biosynthesis, enzymatic in vitro synthesis of branched polylactosamines has advanced rapidly in recent times. Starting from GlcNAc, a series of seven successive in vitro reactions catalyzed by β4GalTI, iGnT, dIGnT, β4GalTI, iGnT, dIGnT and β4GalTI, respectively, with intermittent chromatographic isolation of each intermediary reaction product in pure form, recently yielded the tetradecasaccharide **A** of figure 6 [66]. Treatment of saccharide **A** further with UDP-Gal and α3GalT from bovine thymus gave the octadecameric saccharide **B** of figure 6. Treatment of saccharide **A** with UDP-GlcNAc and iGnT activity of human serum in turn gave the octadecameric saccharide **C** of figure 6. Saccharide B inhibited the binding of murine sperm to murine eggs with and ID_{50} of 2.7 μM, whereas saccharide **C** was not inhibitory [67]. Recent data of Johnston et al. [68], suggest that a variant of saccharide **B**, containing an α3-bonded fucosyl unit at each of the GlcNAcs of the four distal Galα1-3′LacNAc units, could be a truly potent inhibitor of murine gamete adhesion.

In another set of experiments of Seppo et al. [69], the tetradecasaccharide **A** of figure 6 was treated with CMP-Neu5Ac and human placental microsomes containing α2,3-sialyltransferase activity. The resulting tetrasialo-glycan was isolated by ion-exchange chromatography, was characterized by ^{1}H-NMR spectroscopy and was α3-fucosylated by incubation with GDP-Fuc and partially purified α3/4 fucosyltransferases from human milk. The resulting tetrasialo-tetrafuco-glycan was isolated by HPAE-chromatography on a Carbopac PA-1 column from Dionex, and was identified by ^{1}H-NMR spectroscopy as the tetravalent sialyl Lewis x glycan (glycan **D** in fig. 6). This pure oligosaccharide, consisting of 22 monosaccharide units, proved to be a highly potent inhibitor ($IC_{50} \ll 1$ μM) of lymphocyte binding to inflammation-activated endothelium of rejecting renal and cardiac allografts of rats in *ex vivo* binding assays of the Stamper-Woodruff type [69, 70].

Another tetravalent sialyl Lewis x saccharide (glycan **A** in fig. 7) was also constructed in our laboratory and evaluated as an inhibitor of lymphocyte adhesion to inflammation-activated endothelium [71]. In contrast to the glycans of figure 6, the novel polylactosamine represented a long i-type chain with three LacNAc-branches of I-type; all these branches, and also to the distal LacNAc of the long i-chain, were decorated with an α3′-bonded Neu5Ac and an α3-linked Fuc unit in glycan **A** of figure 7. Despite the distinct structural differences, the novel tetravalent sialyl Lewis x (sLex) glycan was of comparable potency with glycan **D** of figure 6 as an inhibitor of lymphocyte-endothelium binding.

A multiply α3-fucosylated polylactosamine with two long, sialic acid-capped branches (glycan **B** in fig. 7) was also constructed in our laboratory recently [72]. Even this construct proved to be an excellent inhibitor of L-selectin-mediated adhesion of rat lymphocytes to rat endothelium. Interestingly, the IC_{50} value of this glycan at lymph node high endothelium was 50 nM, but at the capillaries of rejecting cardiac allografts the IC_{50} value was only 5 nM. Hence, the presence of this and other saccharide antagonists of L-selectin in the bloodstream may reduce extravasation in lymphocytes at sites of inflammation without severely endangering the normal recirculation of lymphocytes via lymph nodes. This may indicate that the concentration of appropriate ligand saccharides is much smaller in the induced state of inflamed human endothelium (Toppila et al. [73]) than in the constitutional state of the high endothelium of lymph nodes (Rosen [5]).

What makes branched polylactosamines such potent inhibitors of lymphocyte adhesion?

Branched polylactosamine backbones represent scaffolds for oligosaccharide determinants that are recognized by cell surface lectins, e.g. the L-selectin of lymphocytes. The sLex-decorations on the synthetic

polylactosamine backbones represent one type of L-selectin-binding ligands, and are believed to be present as distal decorations in the clustered O-glycans of the mucin domains of plasma membrane glycoproteins (e.g. in the CD34 glycoprotein of lymph node endothelium) that act as the biological counterligands of L-selectin. Interestingly, even the intramolecular 'spacers' between the individual decoration groups (i.e. of the number and the rotational freedom of the individual covalent bonds separating these groups intramolecularly) are probably similar in the mucins of plasma membranes and in the I-branched polylactosamines. Hence, even the possible modes of clustering of the saccharide determinants on flexible polylactosamines may mimic those of membrane mucins. This suggests that the ligand properties of appropriate, multiply decorated I-branched polylactosamines in solution may bear close resemblance to the properties of the mucin domains of appropriate endothelial plasma membrane proteins.

In conclusion, the multiplicity of pertinent clustered decorations on synthetic, branched polylactosamines appears to make these molecules very successful competitive inhibitors of the binding between the clustered lymphocyte L-selectin molecules on the tips of microvilli and the counterligands, e.g. the endothelial CD34 glycoprotein, of the lymph nodes.

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