Glycoconjugate Journal (1995) **12**: 36–44

α1,3-Fucosylation of branched blood group I-type oligo-(*N*-acetyllactosamino)glycans by human milk transferases is restricted to distal *N*-acetyllactosamine units: The resulting isomers are separated by WGA-agarose chromatography

RITVA NIEMELÄ, JARI NATUNEN, ELINA BROTHERUS, ANNAMARI SAARIKANGAS and OSSI RENKONEN*

Institute of Biotechnology and Department of Biochemistry, University of Helsinki, P.O.Box 45, FIN-00014 University of Helsinki, Finland.

Received 19 April 1994, revised 15 July 1994.

A partially purified preparation of α1,3-fucosyltransferase(s) from human milk was used to [^{14}C]fucosylate oligosaccharides containing Galβ1-4GlcNAc units. Substitution of *N*-acetyllactosamine at position 3′ with a β-linked *N*-acetylglucosamine enhanced the reactivity of the acceptor, whereas similar substitution at position 6′ was inhibitory. Thus, the trisaccharide GlcNAcβ1-6Galβ1-4GlcNAc (**5**), the branched tetrasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (**11**) and the triply branched decasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3[GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-6]Galβ1-4GlcNAc (**26**) gave remarkably poor yields of α1,3-fucosylated products in comparison to GlcNAcβ1-3Galβ1-4GlcNAc (**3**). β1,4-Galactosyl derivatives of **5** and **11**, however, gave good yields of α1,3-fucosylated products, but the fucosylation was restricted to the distal *N*-acetyllactosamine units of Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc (**16**), Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc (**18**) and also in Galα1-3Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc (**22**). Immobilized wheat germ agglutinin (WGA), possessing high affinity for **16** [1], revealed no affinity for the fucosylated derivative Galβ1-4(Fucα1-3)GlcNAcβ1-6Galβ1-4GlcNAc (**17**). The isomeric heptasaccharides Galβ1-4(Fucα1-3)GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc (**19**) and Galβ1-4GlcNAcβ1-3[Galβ1-4(Fucα1-3)GlcNAcβ1-6]Galβ1-4GlcNAc (**20**) were readily separated from each other on WGA-agarose, and so were the isomeric nonasaccharides Galα1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc (**23**) and Galα1-3Galβ1-4GlcNAcβ1-3[Galα1-3Galβ1-4(Fucα1-3)GlcNAcβ1-6]Galβ1-4GlcNAc (**24**).

Keywords: α1,3-fucosyltransferase(s), human milk, Lewis x, WGA-agarose chromatography, isomer separation

Introduction

The Lewis x determinant Galβ1-4(Fucα1-3)GlcNAc, in appropriately sialylated and/or sulphated form [2], is a ligand of E-, L- and P-selectins, mediating 'physiological' adhesions between leukocytes and endothelium [3–5], 'erroneous' recognition between cancer cells and endothelium [6], and participating in angiogenesis [7]. The Lewis x itself is a low affinity ligand of E- and P-selectins [8,9], and it participates in homotypic sugar-sugar interactions with other Lewis x chains in the presence of Ca^{2+} [10] in a process which appears to be responsible for compaction of morula stage embryos [11]. Besides the adhesive functions, Lewis x appears to possess signalling properties in regulation of T-cells [12]. All these biological roles of Lewis x and sialyl Lewis x may be related to their relatively rigid structures in aqueous solutions [13–15].

In the present report, we describe synthesis of several pure oligo-(*N*-acetyllactosamino)glycans of blood group I-type, carrying Lewis x determinants and mid chain Galβ1-4(Fucα1-3)GlcNAc sequences. The synthesis consisted of α1,3-fucosylation of well defined type 2 acceptors by a partially purified preparation of human milk α1,3-fucosyltransferase(s), followed by chromatography of the products. Particularly valuable separations were effected by affinity chromatography on immobilized wheat germ agglutinin (WGA), which separated several isomeric pairs of glycans from each other. The results

*To whom correspondence should be addressed.

revealed large differences in the reactivity of different types of *N*-acetyllactosamine residues of oligosaccharide acceptors: While unsubstituted *N*-acetyllactosamine units and those monosubstituted at position 3′ reacted well, the glycans containing *N*-acetyllactosamine residues substituted at position 4′ or 6′ were poor acceptors. Galactosylated GlcNAc residues were required for the reaction whereas unsubstituted GlcNAc residues at non-reducing termini were not acceptors. Only distal *N*-acetyllactosamine residues reacted in branched acceptors; the internal branching *N*-acetyllactosamine units were not appreciably fucosylated.

Materials and methods

The oligosaccharides used as acceptors for fucosyltransferase reactions The saccharides **1**, **6** and **7** (for structures, see Table 1) were purchased from Sigma, USA; and **8** from Oxford Glycosystems, UK. The synthesis of following saccharides has been described: **3** and **11** [16], **5** [17], **12** and **16** [18], **24** [19]. **10** was synthesized by incubating Galβ1-4GlcNAc (100 μg), CMP-NeuAc (300 μg; Sigma, USA) and CMP-[^{14}C]NeuAc (0.05 μCi; Amersham, UK) with α2,6-sialyltransferase (5 mU; E.C. 2.4.99.1; Boehringer, Germany) in 50 mM Na-cacodylate buffer, pH 6.0 containing 50 mM NaCl, for 18 hours at 37°C [20]. After heating at 100°C for 5 min, **10** was isolated in a Mono Q HR 5/5 anion-exchange column (Pharmacia, Sweden) using a NaCl gradient as described in [21]; the saccharide was finally desalted in a column of Sephadex G-10 (Pharmacia, Sweden). **22** was synthesized by α1,3-galactosylating **18** as described in [22]. **26** was synthesized by β1,6-*N*-acetylglucosaminylation of GlcNAcβ1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-3Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc [23] as described in a separate study (Seppo A, Penttilä L, Niemelä R, Renkonen O, Keane A, unpublished experiments).

α1,3-Fucosyltransferase reactions The transferase reactions were performed with a partially purified α1,3/(α1,4)-fucosyltransferase from human milk essentially as described [24–26]. The enzyme preparation was free of detectable amounts of β-galactosidase and β-*N*-acetylhexosaminidase activities. The acceptor saccharide (1.0 nmol) and 750 pmol GDP-[^{14}C]fucose (the specific activities used were 239, 278, 292 or 333 mCi mmol^{-1}, depending on the batch; Amersham, UK) were dissolved in 25 μl of 0.1 M MOPS (Sigma)-NaOH, pH 7.5, 0.2 M NaCl, 0.01 M $MnCl_2$ and 1.0 mg ml^{-1} BSA; 15 μl of water and 10 μl of the enzyme (8.8 μU) were added. The mixture was incubated for 24 h at 37°C, heated at 100°C for 3 min, and desalted as described below. Glycerol and free [^{14}C]fucose were removed by paper chromatography, run overnight with solvent A (see below). The individual glycans were studied in only one or a few experiments (see Table 2), but glycan **3** was included in each of the eleven separate series of reactions, yielding 221 +/– 52 pmol of glycan **4**.

Glycosidase reactions Hydrolysis with β-galactosidase (jack beans, EC 3.2.1.23; Sigma, USA) was performed as described [27]. Concurrent hydrolysis catalysed by β-galactosidase (jack beans) and β-*N*-acetylhexosaminidase (jack beans, EC.3.2.1.30; Sigma, USA) was performed in 30 μl 50 mM Na-citrate buffer, pH 4.0, where 100 mU of β-galactosidase and 150 mU of β-*N*-acetylhexosaminidase were added; incubation was at 37°C for 16 h and the reaction was stopped by heating at 100°C for 3 min. Hydrolysis with α-galactosidase (green coffee beans, EC 3.2.1.22; Sigma, USA) was performed in a tube which contained the dry sample, 100 μl citrate-phosphate buffer, pH 6.6, and 1 U α-galactosidase. The mixture was incubated for 7 h at 37°C and heated at 100°C for 3 min. Defucosylation by almond meal 1 α-fucosidase (EC 3.2.1.111; Oxford Glycosystems, UK) was performed in 10 μl of 50 mM Na-acetate buffer, pH 5.0, 0.7 mg ml^{-1} BSA, by 2 μU of enzyme for 24 h at 37°C and terminated by heating at 100°C for 3 min.

Chromatographic methods Descending paper chromatography was performed as described [28], with the upper phase of n-butanol:acetic acid:water 4:1:5 (v/v) (solvent A) or with n-butanol:acetic acid:water 10:3:7 (v/v) (solvent F). The mobilities of saccharides are presented in relation to galactose, lactose, maltooligosaccharides (Sigma, USA) and isomaltooctaose (BioCarb, Sweden). Distribution of radioactivity on the chromatograms was measured as described [28], using Opti-Scint from Wallac (Turku, Finland) as the scintillant. Unlabelled markers were stained by silver nitrate [29].

WGA-affinity chromatography was carried out as described [30], using columns of low WGA content (1.65 mg WGA per ml agarose) and high WGA content (9.6 mg WGA per ml agarose). Saccharides were eluted from the column of high WGA content with 10 mM phosphate buffer, pH 7.1, containing 0.15 M NaCl and 0.02% NaN_3.

Desalting All enzymatic reaction mixtures and pooled saccharides were desalted by passage through beds of Dowex AG-1 (AcO^-) and Dowex AG-50W (H^+) ion exchangers. Neutral saccharides were eluted with water, and charged saccharides with 0.5 M acetic acid as in [31].

Results

The structures and numbering of the saccharides studied in the present experiments are presented in Table 1.

Relative yields of fucosylated products obtained in partial reactions with a partially purified preparation of α1,3-fucosyltransferase(s) from human milk A number of type 2 oligosaccharide acceptors were incubated with GDP-[^{14}C]fucose and a partially purified preparation of α1,3-fucosyltransferase(s) of human milk under conditions effecting only partial reactions. The data collected in Table 2 show that substitution at position 3′ enhances the reactivity of *N*-acetyllactosamine. Thus, GlcNAcβ1-3Galβ1-4GlcNAc (**3**) gave a

Table 1. Structures and numbering of the saccharides discussed in this study.

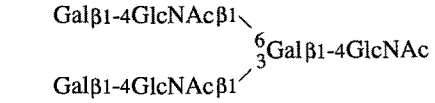
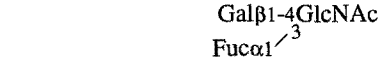
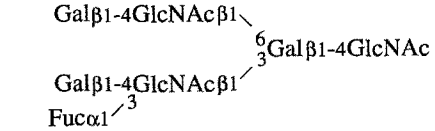
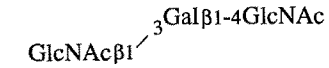
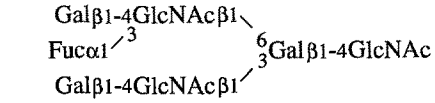
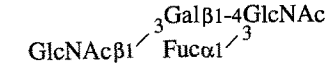
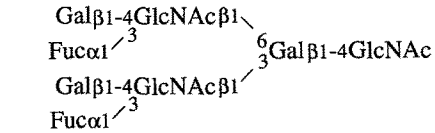
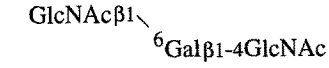
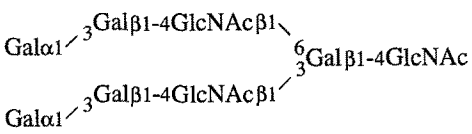
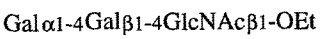
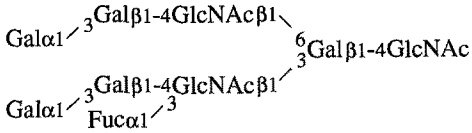
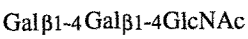
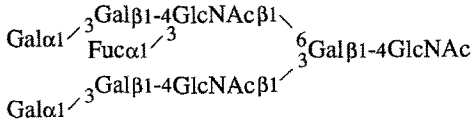
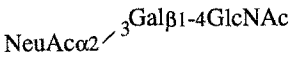
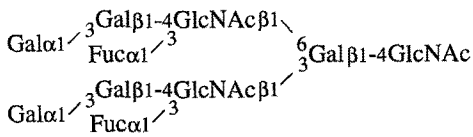
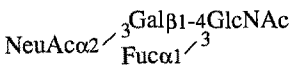
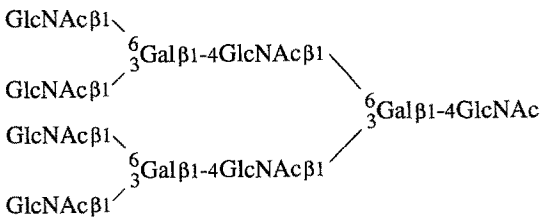
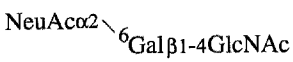
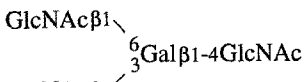
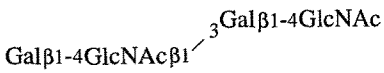
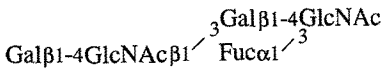
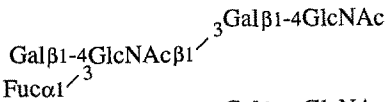
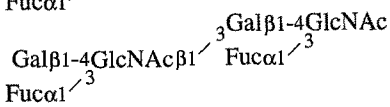
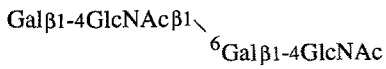
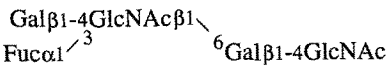
No	Saccharide	No	Saccharide
1		**18**	
2		**19**	
3		**20**	
4		**21**	
5		**22**	
6		**23**	
7		**24**	
8		**25**	
9		**26**	
10			
11			
12			
13			
14			
15			
16			
17			

Table 2. Relative amounts of fucosylated products formed from type 2 acceptors with a partially purified preparation of α1,3-fucosyltransferase(s) of human milk.

No	Saccharide	Relative amount of product formed [1]
1	Galβ1-4GlcNAc	29
3	3Galβ1-4GlcNAc GlcNAcβ1⁄	100
5	GlcNAcβ1╲ 6Galβ1-4GlcNAc	2
6	Galα1-4Galβ1-4GlcNAcβ1-OEt	3
7	Galβ1-4Galβ1-4GlcNAc	2
8	NeuAcα2⁄3Galβ1-4GlcNAc	55
10	NeuAcα2╲ 6Galβ1-4GlcNAc	0
11	GlcNAcβ1╲ 6 3Galβ1-4GlcNAc GlcNAcβ1⁄	1
12	3Galβ1-4GlcNAc Galβ1-4GlcNAcβ1⁄	132
16	Galβ1-4GlcNAcβ1╲ 6Galβ1-4GlcNAc	67
18	Galβ1-4GlcNAcβ1╲ 6 3Galβ1-4GlcNAc Galβ1-4GlcNAcβ1⁄	107
22	Galα1⁄3Galβ1-4GlcNAcβ1╲ 6 3Galβ1-4GlcNAc Galα1⁄3Galβ1-4GlcNAcβ1⁄	202
26	GlcNAcβ1╲ 6 3Galβ1-4GlcNAcβ1╲ GlcNAcβ1⁄ 6 3Galβ1-4GlcNAc GlcNAcβ1╲ 6 3Galβ1-4GlcNAcβ1⁄ GlcNAcβ1⁄	4

[1] Note: Relative amounts of products represent total incorporation of the [^{14}C]fucose to the acceptor, comparing the incorporated activity of each acceptor to that of **3**. Reactions with **3** were performed 11 times (standard), with **12** and **22** three times, with **1** and **18** twice and all others only once. With saccharides **12**, **16**, **18** and **22** the molar amounts of the difucosyl products represented 4%, ≪1%, 7% and 5% of the fucosylated glycans, respectively. The absolute amount of [^{14}C]fucose transferred to **3** was 221 pmol.

three times higher yield than the unsubstituted Galβ1-4GlcNAc (**1**), NeuAcα2-3Galβ1-4GlcNAc (**8**) was a remarkably good acceptor, and also substitution by α1,3-bonded galactose residues doubled the yield as shown by compounds **18** and **22**.

In contrast, substitution of *N*-acetyllactosamine at position 6' inhibits the acceptor reactivity. Thus, the trisaccharide GlcNAcβ1-6Galβ1-4GlcNAc (**5**) gave a very poor yield and the trisaccharide NeuAcα2-6Galβ1-4GlcNAc (**10**) was virtually unreactive, confirming the previous observations of Paulson *et al.* [32].

In di-substituted *N*-acetyllactosamine residues, the inhibitory effect of substitution at position 6′ overruled the stimulatory influence of substitution at position 3′. Consequently, the branched tetrasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (**11**) and the triply branched decasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3[GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-6]Galβ1-4GlcNAc (**26**) were remarkably poor acceptors.

Substitution at position 4′ was also quite inhibitory: the trisaccharides Galα1-4Galβ1-4GlcNAcβ1-OEt (**6**) and Galβ1-4Galβ1-4GlcNAc (**7**) were very poor acceptors.

Table 2 shows that even proximal substitution at position 1 of *N*-acetyllactosamine can enhance fucosylation. Accordingly, compound **16**, a di-*N*-acetyllactosamine saccharide reacting almost solely at the non-reducing unit (see below), was much more reactive than *N*-acetyllactosamine (**1**). In the same vein, compound **18** was four times more reactive than *N*-acetyllactosamine, although it reacted only with its two units at the non-reducing area. These findings are compatible with the notion that the reducing end GlcNAc of *N*-acetyllactosamine may react poorly in α-configuration.

Table 2 supports also the findings of degradation experiments described below, establishing that β1,4-galactosylation of GlcNAc units is required for α1,3-fucosylation and that unsubstituted GlcNAc residues at the nonreducing area are not reactive. Consequently, compounds **5**, **11**, and **26** were poor acceptors, while the β1,4-galactosylated species **16** and **18** were quite reactive.

Product characterization The fucosylated product obtained from Galβ1-4GlcNAc (**1**) was the Lewis x trisaccharide Galβ1-4(Fucα1-3)GlcNAc (**2**) as established by 1D and 2D ^{1}H-NMR-spectroscopy at 500 MHz, and by cleavage with almond meal 1 α-fucosidase in a separate study [26].

The product obtained from the trisaccharide GlcNAcβ1-3Galβ1-4GlcNAc (**3**) revealed on paper chromatography only mono-[^{14}C]fucosylated saccharide(s) (R_{MT} = 0.83, R_{MTe} = 1.32; solvent A), which were cleaved to the extent of 97% by treatment with β-*N*-acetylhexosaminidase into Galβ1-4([^{14}C]Fucα1-3)GlcNAc (R_{Lac} = 0.93, R_{MT} = 1.26; solvent A). Thus, the product was identified as GlcNAcβ1-3Galβ1-4([^{14}C]Fucα1-3)GlcNAc (**4**), implying that the GlcNAc at the non-reducing end of **3** had not reacted. The fucosylation products from the trisaccharide GlcNAcβ1-6Galβ1-4GlcNAc (**5**) and from the branched tetrasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (**11**) were not properly characterized.

Two monofucosylated products were obtained from the tetrasaccharide Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (**12**); they were Galβ1-4GlcNAcβ1-3Galβ1-4([^{14}C]Fucα1-3)GlcNAc (**13**) and Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAc (**14**) as established in degradation and NMR experiments of a separate study (Niemelä R, Natunen J, Penttilä L, Seppo A, Ruohtula T, Renkonen O, unpublished observations); a small amount of the difucosyltetrasaccharide (**15**) was also formed.

In contrast, the products from the isomeric tetrasaccharide Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc (**16**) represented mono-[^{14}C]fucosylated saccharides as revealed by paper chromatography (Fig. 1). Most of this material (97%) resisted β-galactosidase treatment, but was cleaved to the extent of 53% by a treatment with almond 1 α-fucosidase. These data established that it represented Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-6Galβ1-4GlcNAc (**17**) [33–35]. The absence of galactosidase-sensitive monofucosylated product and also the absence of difucosylated product suggested that the reducing end *N*-acetyllactosamine unit of **16** was relatively resistant to fucosylation.

The [^{14}C]fucosylation products obtained from the branched hexasaccharide Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc (**18**) represented monofucosylhexasaccharides and difucosylhexasaccharides as shown by paper chromatography (Fig. 2A). The monofucosylhexasaccharides consisted of a mixture of Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc (**19**) (62%) and Galβ1-4GlcNAcβ1-3[Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-6]Galβ1-4GlcNAc (**20**) (38%). This was shown by degradation of the mixture concurrently with β-galactosidase and β-*N*-acetylhexosaminidase, and by subsequent chromatographic separation of the monofucosyltetrasaccharides Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAc (**14**) and

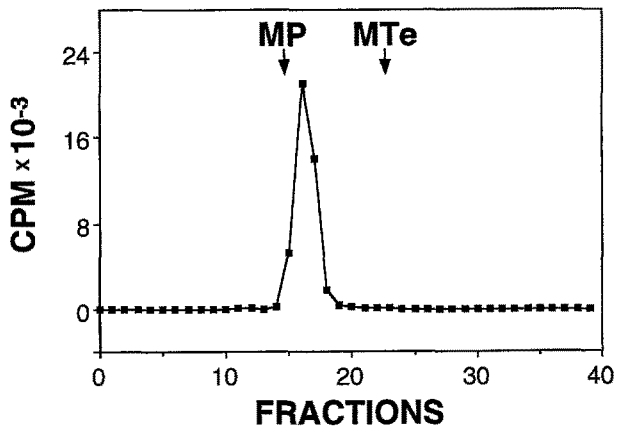


Figure 1. *Paper chromatogram related to α1,3-[^{14}C]fucosylation of the tetrasaccharide **16**.* Products from the [^{14}C]fucosylation reaction. The peak represents monofucosyl compound(s), which was shown to be mainly the saccharide **17**. Solvent A, 119 h. MTe and MP show the positions for maltotetraose and -pentaose markers, respectively.

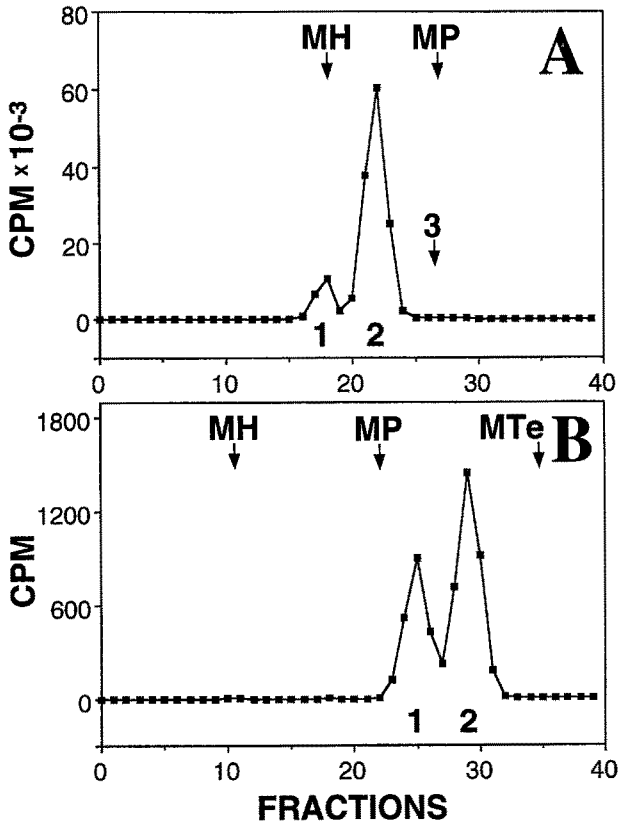


Figure 2. *Paper chromatograms related to α1,3-[^{14}C]fucosylation of the branched hexasaccharide **18**.* Panel **A**. Products from the [^{14}C]fucosylation reaction. Peak 1 represents difucosyl derivatives, while peak 2 consists of monofucosyl compounds; the unreacted acceptor runs at the position of arrow 3. Solvent F, 65 h. MP and MH show the positions for maltopentaose and -heptaose markers, respectively. Panel **B**. Products from a digestion of the monofucosyl compounds from peak 2/Fig. 2A, with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase. Peak 1 migrated like the synthetic pentasaccharide **17**, while peak 2 occupied the position of the synthetic isomer **14**. Solvent A, 7 days. Markers as in panel A, in addition to MTe, maltotetraose.

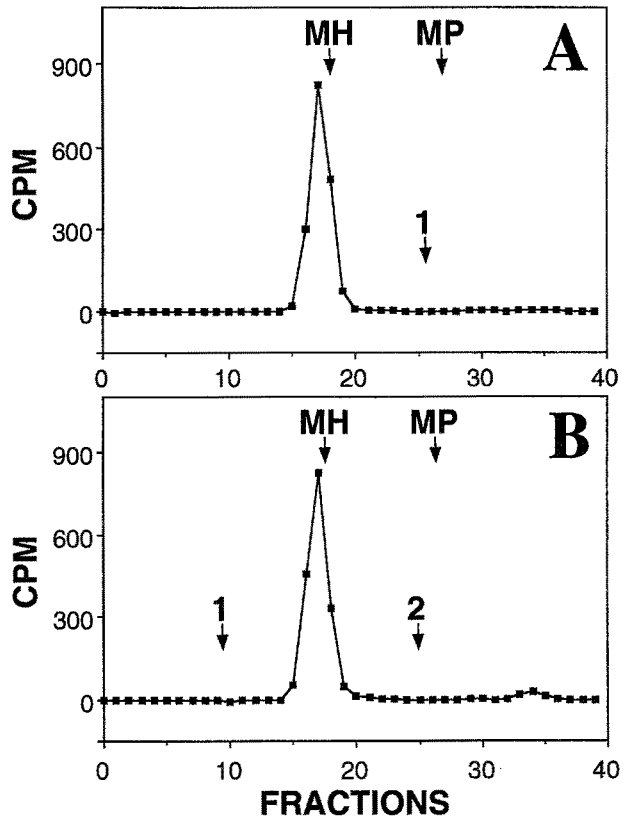


Figure 3. *Paper chromatograms related to the structural analysis of difucosylsaccharides **21** and **25**.* Panel **A**. Products from concomitant digestion of peak 1/Fig. 2A with β-galactosidase and β-*N*-acetylhexosaminidase. The product peak runs at the position of the unchanged substrate; arrow 1 shows the position of **15**. Solvent F, 63 h. Markers as in Fig. 2A. Panel **B**. Products from digestion of peak 1/Fig. 4A with α-galactosidase and then concomitantly with β-galactosidase and β-*N*-acetylhexosaminidase. The product runs at the position of **21**; arrow 1 shows the position of original difucosyloctasaccharide; arrow 2 shows the position of **15**. Solvent F, 63 h. Markers as in Fig. 2A.

Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-6Galβl-4GlcNAc (**17**) of linear backbones (Fig. 2B).

The fraction of putative difucosylhexasaccharides from peak 1/Fig. 2A was completely resistant to a concurrent cleavage by β-galactosidase and β-*N*-acetylhexosaminidase. Thus, the digest contained only the unchanged substrate migrating just behind the maltoheptaose marker; practically no radiolabel was present in the area of the difucosyltetrasaccharide **15**, a degradation product expected from the difucosylhexasaccharide Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4([^{14}C]Fucα1-3)GlcNAc that has one arm unprotected by the fucosyl residue (Fig. 3A). These data suggest that only the difucosylhexasaccharide isomer **21** had been formed during [^{14}C]fucosylation of **18**.

The [^{14}C]fucosylation products obtained from the branched octasaccharide Galα1-3Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc (**22**) represented monofucosyloctasaccharides and difucosyloctasaccharides as shown by paper chromatography (Fig.4A). The monofucosyloctasaccharides consisted of a mixture of Galα1-3Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc (**23**) (61%) and Galα1-3Galβ1-4GlcNAcβ1-3[Galα1-3Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-6]Galβ1-4GlcNAc (**24**) (39%). This was shown by a degradation with α-galactosidase and then concomitantly with β-galactosidase and β-*N*-acetylhexosaminidase, and by subsequent chromatographic identification of the linear monofucosyltetrasaccharides **14** and **17** (Fig. 4B).

The putative difucosyloctasaccharides from peak 1/Fig. 4A lost only the two α-galactose residues in a cleavage experiment with α-galactosidase followed by a concurrent hydrolysis by β-galactosidase and β-*N*-acetylhexosaminidase. Thus, the digest contained only the saccharide **21** migrating just behind maltoheptaose marker; practically no radiolabel was

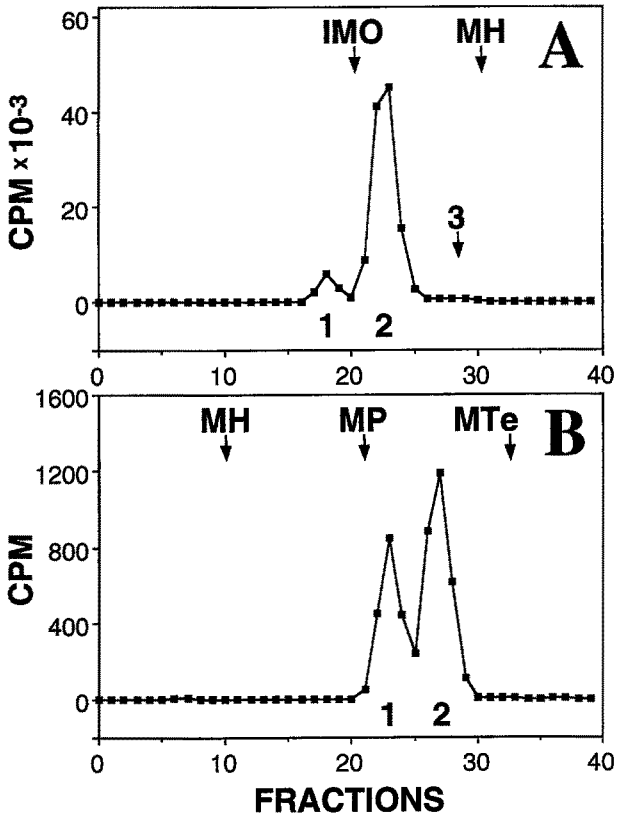


Figure 4. *Paper chromatograms related to α1,3-[^{14}C]fucosylation of the branched octasaccharide* ***22***. Panel **A**. Products from the [^{14}C]fucosylation reaction. Peak 1 represents difucosyl derivatives, while peak 2 consists of monofucosyl compounds; the unreacted acceptor runs at the position of the arrow 3. Solvent F, 72 h. MH and IMO show the positions for maltoheptaose and isomaltooctaose markers, respectively. Panel **B**. Products from a digestion of the monofucosyl compounds from peak 2/Fig. 4A, first with α-galactosidase and then concomitantly with β-galactosidase and β-*N*-acetylhexosaminidase. Peak 1 chromatographed like **17**, while peak 2 migrated like **14**. Solvent A, 7 days. Markers as in Fig. 2B.

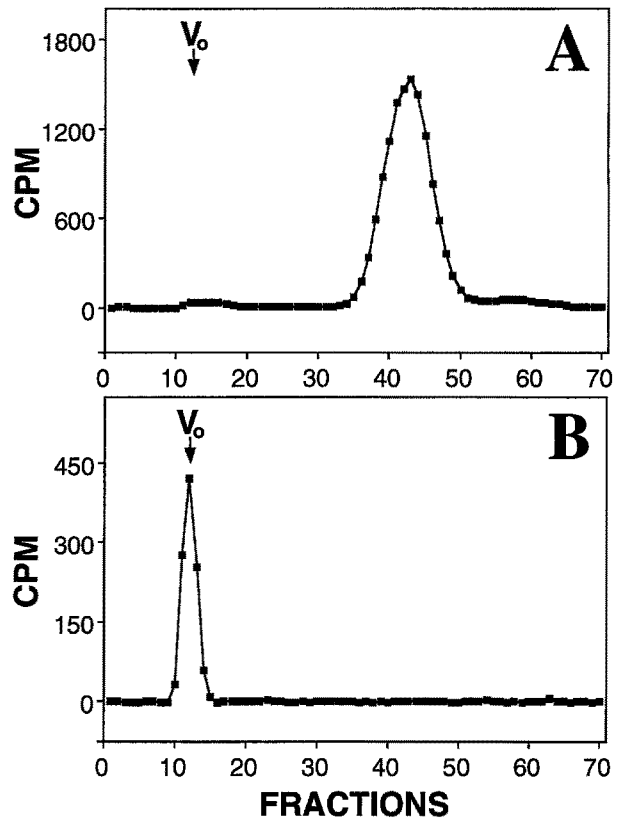


Figure 5. *WGA-agarose chromatograms in the column of low WGA-content*. Panel **A**. Backbone saccharide **16**. Panel **B**. Fucosylated saccharide **17**.

present in the area of the difucosyltetrasaccharide **15**, a degradation product expected from the difucosyloctasaccharide Galα1-3Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)Galβ1-4([^{14}C]Fucα1-3)GlcNAc that has one arm completely unprotected by the fucosyl residue (Fig. 3B). These data suggest that only the difucosyloctasaccharide isomer **25** had been formed during [^{14}C]fucosylation of **22**.

WGA-chromatography of the fucosylated oligosaccharides
The linear backbone tetrasaccharide Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc (**16**) revealed a considerable affinity to WGA, as shown by its retardation by the WGA-agarose column of low WGA content (Fig. 5A) [1]. In contrast, the monofucosylated derivative Galβ1-4([^{14}C]Fucα1-3)GlcNAc β1-6Galβ1-4GlcNAc (**17**) possessed no affinity to WGA as shown in Fig. 5B.

In a separate column, containing a high concentration of WGA, the mixture of monofucosylhexasaccharides **19** (62%) and **20** (38%) was resolved into the two components as shown in Fig. 6A. Peak 1 contained 40% of the total radioactivity while the slowly eluting peak 2 contained 58% of the label. Accordingly, the former represented **20** while the latter contained the isomer **19**. This notion was confirmed also by degradation experiments: peak 1 material gave pentasaccharide **17** when treated with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase, while peak 2 gave the isomeric pentasaccharide **14** (data not shown).

Also the mixture of the isomeric monofucosyloctasaccharides **23** (61%) and **24** (39%) could be readily separated in the WGA-agarose column of high WGA-content (Fig. 6B). The early eluting peak 1 (42% of label) is taken to represent the isomer **24**, while the peak 2 (58% of label) is identified as the isomeric **23**.

Discussion

The present data reveal that substitution of *N*-acetyllactosamine units controls acceptor reactivity in α1,3-fucosyltransferase reactions catalysed by an enzyme mixture from human milk. The plasma type (EC 2.4.1.152) and the Lewis type enzymes (EC 2.4.1.65), also known as FT VI, and FT III,

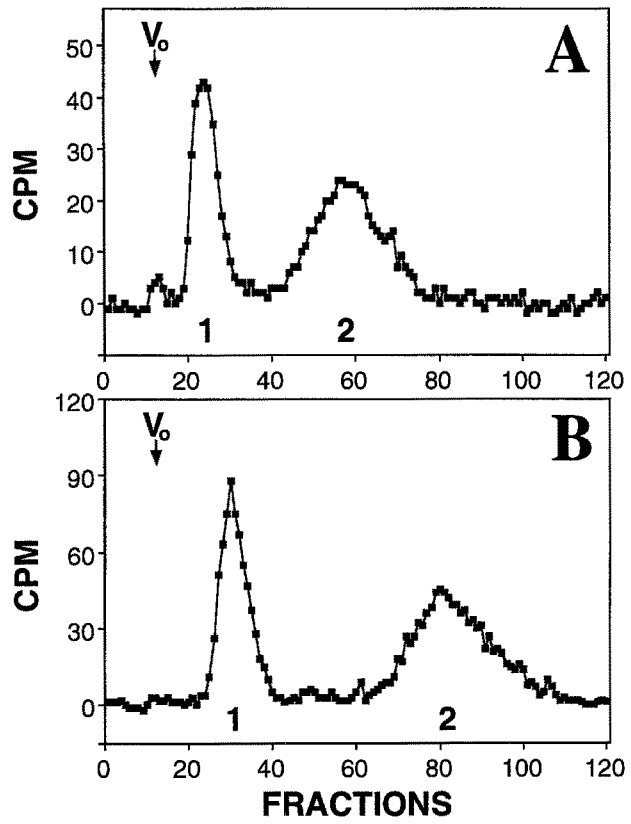


Figure 6. *WGA-agarose chromatograms in the column of high WGA-content.* Panel **A**. An aliquot from peak 2/Fig. 2A. Peak 1 proved to be monofucosylhexasaccharide **20** and peak 2 the isomer **19**. Panel **B**. An aliquot from peak 2/Fig. 4A. Peak 1 represents **24** and peak 2 the isomer **23**.

respectively, were probably present, but the contribution of the Lewis enzyme appeared not to be important, because the enzyme reacted rather poorly with Galβ1-3GlcNAc, the preferred acceptor of the Lewis enzyme. No α1,2-fucosyltransferase activity was present as judged from chromatographic and NMR experiments performed with the fucosylated product from *N*-acetyllactosamine [26].

Substitution of *N*-acetyllactosamine at position 3′ by an α-linked galactose, *N*-acetylneuraminic acid or by a β-linked *N*-acetylglucosamine enhanced the acceptor reactivity. In contrast, substitutions at positions 4′or 6′ were found to be strongly inhibitory. Paulson *et al.* [32] have shown previously that α2,6′-sialylation of *N*-acetyllactosamine residues inhibits subsequent fucosylation by the human milk enzymes. It appears that the distal galactose residue, critically needed for α1,3-fucosylation, is bound to the enzyme by the hydroxyl groups at positions 4′ and 6′. Substituents at position 1 of *N*-acetyllactosamine appeared not to be inhibitory and in compounds **16** and **18** they were clearly stimulatory. The large differences in the reactivities of substituted *N*-acetyllactosamines with the milk enzyme suggest that some of the acceptors used could be helpful in characterizing purified α1,3-fucosyltransferases and recombinant transferases.

In contrast to the poorly reacting glycans **5** and **11**, the β1,4-galactosylated derivatives **16** and **18** were good acceptors, and so was the branched octasaccharide Galα1-3Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc (**22**). However, degradation experiments established that the fucosylated products from the galactosylated acceptors carried the fucose residues in the distal branches, while the 'branching', or 6′-substituted, *N*-acetyllactosamine residue at the reducing end had not reacted appreciably. The hexasaccharide **18** was fucosylated faster at the 1,3-linked arm than at the 1,6-branch, while in a separate study lacto-*N*-neohexaose was α1,3-fucosylated slightly faster at the 1,6-branch [26]. Analogous differences between the two hexasaccharides, and also between **18** and its truncated form missing the reducing end GlcNAc have been reported before [17,36].

Immobilized WGA had a central role in the present experiments, because of its ability to separate fucosylated glycans. The branched difucosylhexasaccharide **21** revealed no affinity to WGA-agarose (not shown), while the related monofucosylhexasaccharide Galβ1-4GlcNAcβ1-3[Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-6]Galβ1-4GlcNAc **20** showed a weak affinity, and the isomer **19** possessed a strong affinity. The affinity differences were large enough to allow the separation of the three components in a single run on a small column of WGA-agarose. Similar data were obtained on monofucosyl derivatives of the branched octasaccharide **22**, extending the applicability of WGA-agarose chromatography in the study of isomeric fucosylsaccharides. Our data imply that α1,3-fucosylation of the individual *N*-acetyllactosamine residues within a large oligo-(*N*-acetyllactosamino)glycan reduces the WGA-affinity of the parent compound to a different degree. In particular prominent reductions in the affinity are effected by fucosylation of the 1,6-linked arms of the branched saccharides **18** and **22**.

The easy access of pure isomers of branched Lewis x-related glycans that is provided by WGA-agarose chromatography should prove helpful for further development of adhesion- and signal-oriented studies with these saccharides.

Acknowledgements

The work was supported in part by grants from the University of Helsinki and the Finnish Academy.

References

1. Renkonen O, Penttilä L, Niemelä R, Vainio A, Leppänen A, Helin J, Seppo A, Makkonen A, Maaheimo H (1991) *Carbohydr Res* **213**: 169–83.
2. Yuen C-T, Bezouska K, O'Brien J, Stoll M, Lemoine R, Lubineau A, Kiso M, Hasegawa A, Bockovich NJ, Nicolaou KC, Feizi T (1994) *J Biol Chem* **269**: 1595–98.
3. Phillips ML, Nudelman E, Gaeta FCA, Perez M, Singhal AK, Hakomori S-I, Paulson JC (1990) *Science* **250**: 1130–32.
4. Polley MJ, Phillips ML, Wayner E, Nudelman E, Singhal AK, Hakomori S-I, Paulson JC (1991) *Proc Natl Acad Sci USA* **88**: 6224–28.

5. Butcher EC (1991) *Cell* **67**: 1033–36.
6. Matsusako T, Muramatsu H, Shirahama T, Muramatsu T, Ohi Y (1991) *Biochem Biophys Res Commun* **181**: 1218–22.
7. Nguyen M, Strubel NA, Bischoff J (1993) *Nature* **365**: 267–69.
8. Larsen E, Palabrica T, Sajer S, Gilbert GE, Wagner DD, Furie BC, Furie B (1990) *Cell* **63**: 467–74.
9. Larkin M, Ahern TJ, Stoll MS, Shaffer M, Sako D, O'Brien J, Yuen C-T, Lawson AM, Childs RA, Barone KM, Langer-Safer PR, Hasegawa A, Kiso M, Larsen GR, Feizi T (1992) *J Biol Chem* **267**: 13661–68.
10. Hakomori S-I (1993) *Biochem Soc Trans* **21**: 583–95.
11. Fenderson BA, Zehavi U, Hakomori S-I (1984) *J Exp Med* **160**: 1591–96.
12. Velupillai P, Harn DA (1994) *Proc Natl Acad Sci USA* **91**: 18–22.
13. Wormald MR, Edge CJ, Dwek RA (1991) *Biochem Biophys Res Commun* **180**: 1214–21.
14. Ball GE, O'Neill RA, Schulz JE, Lowe JB, Weston BW, Nagy JO, Brown EG, Hobbs CJ, Bednarski MD (1992) *J Am Chem Soc* **114**: 5449–51.
15 Rutherford TJ, Spackman DG, Simpson PJ, Homans SW (1994) *Glycobiology* **4**: 59–68.
16. Seppo A, Penttilä L, Makkonen A, Leppänen A, Jäntti J, Helin J, Renkonen O (1990) *Biochem Cell Biol* **68**: 44–53.
17. Renkonen O, Niemelä R, Leppänen A, Maaheimo H, Seppo A, Penttilä L, Vilkman A (1991) *Glycoconjugate J* **8**: 368–75.
18. Renkonen O, Penttilä L, Niemelä R, Leppänen A (1991) *Glycoconjugate J* **8**: 376–80.
19. Renkonen O, Helin J, Vainio A, Niemelä R, Penttilä L, Hilden P (1990) *Biochem Cell Biol* **68**: 1032–36.
20. Weinstein J, de Souza-e-Silva U, Paulson JC (1982) *J Biol Chem* **257**: 13845–53.
21. Damm JB, Kamerling JP, van Dedem GWK, Vliegenthart JFG (1987) *Glycoconjugate J* **4**: 129–44.
22. Seppo A, Penttilä L, Leppänen A, Maaheimo H, Neimelä R, Helin J, Wieruszeski J-M, Renkonen O (1994) *Glycoconjugate J*, **11**: 217–25.
23. Vilkman A, Niemelä R, Penttilä L, Helin J, Leppänen A, Maaheimo H, Lusa S, Renkonen O (1992) *Carbohydr Res* **226**: 155–74.
24. Eppenberger-Castori S, Lötscer H, Finne J (1989) *Glycoconjugate J* **6**: 101–14.
25. Prieels J-P, Monnom D, Dolmans M, Beyer TA, Hill RL (1981) *J Biol Chem* **256**: 10456–63.
26. Natunen J, Niemelä R, Penttilä L, Seppo A, Ruohtula T, Renkonen O (1994) *Glycobiology* **4**: 577–83.
27. Renkonen O, Helin J, Penttilä L, Maaheimo H, Neimelä R, Leppänen A, Seppo A, Hård K (1991) *Glycoconjugate J* **8**: 361–67.
28. Renkonen O, Penttilä L, Makkonen A, Niemelä R, Leppänen A, Helin J, Vainio A (1989) *Glycoconjugate J* **6**: 129–40.
29. Trevelyan WE, Procter DP, Harrison JS (1950) *Nature* **166**: 444–45.
30. Renkonen O, Mäkinen P, Hård K, Helin J, Penttilä L (1988) *Biochem Cell Biol* **66**: 449–53.
31. Koszdin KL, Bowen BR (1992) *Biochem Biophys Res Comm* **187**: 152–57.
32. Paulson JC, Prieels J-P, Galsgow LR, Hill RL (1978) *J Biol Chem* **253**: 5617–24.
33. Arakawa M, Ogata S-I, Muramatsu T, Kobata A (1974) *J Biochem* **75**: 707–14.
34. Ogata-Arakawa M, Muramatsu T, Kobata A (1977) *Arch Biochem Biophys* **181**: 353–58.
35. Yamashita K, Tachibana Y, Kobata A (1977) *J Biol Chem* **252**: 5408–11
36. Helin J, Seppo A, Leppänen A, Penttilä L, Maaheimo H, Niemelä R, Lauri S, Renkonen O (1993) *FEBS Lett* **335**: 280–84.