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ENZYMATIC SYNTHESIS OF THE α3-GALACTOSYL-LE^x TETRASACCHARIDE: A POTENTIAL LIGAND FOR SELECTIN-TYPE ADHESION MOLECULES

D.H. Joziasse, W.E.C.M. Schiphorst, C.A.M. Koeleman, and D.H. Van den Eijnden

Department of Medical Chemistry, Vrije Universiteit, v.d. Boechorststraat 7, NL-1081BT Amsterdam, The Netherlands

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Summary: Using recombinant UDP-Gal:Galβ1→4GlcNAc α1,3-galactosyltransferase and human milk α1,3-fucosyltransferase the disaccharide Galβ1→4GlcNAc has been converted *in vitro* into a tetrasaccharide product. The product has been characterized by gel filtration chromatography and HPLC and was analyzed using ^{1}H-NMR. Based on NMR spectral data along with the known linkage specificity of the α1,3-galactosyltransferase and the α1,3-fucosyltransferase used, the chromatographic behaviour of the product, and the 1:1 molar ratios of the galactose and fucose residues calculated from incorporated radioactivity, it is concluded that the structure of the tetrasaccharide product is Galα1→3Galβ1→4[Fucα1→3]-GlcNAc. The tetrasaccharide is a non-charged analogue of the sialyl-Le^x determinant that potentially may act as a ligand structure in selectin-mediated cell-cell adhesion.

N-acetyllactosamine (LacNAc) units are known to occur on both N-linked and O-linked glycans of mammalian glycoproteins. In many instances these chains are extended with additional LacNAc units to form polylactosaminoglycans, and usually they are terminated by α2,3- and α2,6-linked sialic acid. In addition, a number of alternative terminal structures have been reported which can be regarded as variations on this common theme. Some of these structures involve a novel combination of elements that may confer a specific biological function. A terminal structure that has received much attention lately is the sialyl-Le^x determinant which combines fucose and sialic acid. Over the last few years it has become clear that sugar chains that are both α2,3-sialylated and α1,3-fucosylated, such as those that contain the sialyl-Le^x determinant, play a major role in cellular adhesion by interacting with the "selectins" [1,2]. In addition, sulfation of a related determinant appears to modulate its selectin binding properties [3].

A non-charged alternative for chain termination by sialic acid is the transfer of an α1,3-galactosyl group to the terminal Gal of the N-acetyllactosamine unit, to produce the structure Galα1→3Galβ1→4GlcNAc-R [4]. This modification occurs in many species of mammals, with the exception of humans, apes and Old World monkeys: The genome of the latter species does not contain a functional α1,3-galactosyltransferase gene [5,6]. Theoretically,

Abbreviations used: Fuc, L-Fucose; NeuAc, N-acetylneuraminic acid; LacNAc, N-acetyllactosamine (Galβ1→4GlcNAc); α1,3-GT, UDP-Gal:Galβ1→4GlcNAc α1,3-galactosyltransferase; HPAEC, High-pH Anion Exchange Chromatography.

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combination of α-linked galactose with other terminal elements might create novel adhesion determinants.

With the long range goal of identifying alternative cell adhesion determinants, we decided to investigate whether *in vitro* enzymatic synthesis of the compound Galα1→3Galβ1→4-[Fucα1→3]GlcNAc is possible. So far, occurrence *in vivo* of this determinant has not been reported. Here we describe the synthesis of the tetrasaccharide at a micromolar scale. Based on the known substrate specificities of the enzymes used an ordered pathway for its synthesis is proposed in which α1,3-galactosylation has to precede α1,3-fucosylation. Our results will help to identify the Galα1→3-Le^x determinant in glycoconjugates *in vivo*. In addition, the availability of the enzymatic product may be useful to address the issue of whether glycoconjugates carrying this structure would have any biological activity such as in cell adhesion.

EXPERIMENTAL PROCEDURES

Materials

N-acetyllactosamine (LacNAc, Galβ1→4GlcNAc) was kindly donated by Dr. Veyrières (Université Paris Sud, Orsay, France), and unlabeled GDP-fucose was a kind gift of Dr. Th. Norberg and H. Lönn (Biocarb AB, Lund, Sweden). UDP-[^{14}C]Gal (300 Ci/mol) and GDP-[^{3}H]fucose (7000 Ci/mol) were from Dupont NEN, and were diluted with the corresponding unlabeled nucleotide sugar to the desired specific radioactivity.

Recombinant α1,3-galactosyltransferase

Recombinant soluble bovine UDP-Gal:Galβ1→4GlcNAc α1,3-galactosyltransferase (α1,3-GT) was produced in *Spodoptera frugiperda* (Sf9) insect cells (to be published elsewhere). Briefly, from a bovine α1,3-GT cDNA [7] a portion was deleted that encoded the amino-terminal tail and the signal-anchor domain, prior to ligation into the transfer vector pVT-Bac [8] (kindly donated by Dr. T. Vernet, Genetic Engineering Section of the Biotechnology Research Institute, Québec, Canada), and the construct was used to generate recombinant baculovirus. The presence of an insect cell signal peptide in the construct resulted in effective secretion of soluble recombinant α1,3-GT by virus-infected cells. The preparation thus obtained contained an enzyme activity of ≈ 85 mU/ml of serum-free growth medium, and was used without further purification for the α-galactosylation of N-acetyllactosamine. Enzyme activity was determined using lactose as the acceptor substrate as previously described [4].

Partially purified fucosyltransferase from human milk

Partly purified human milk α1,3-fucosyltransferase was kindly provided by Dr. Th. de Vries of this laboratory. It was obtained from human milk from healthy lactating mothers which was stored frozen until use. Purification of this enzyme was performed at 4°C, according to published procedures [9,10] with minor modifications. The preparation obtained was purified 225-fold and contained 70 mU of enzyme activity (88% yield) in a volume of 50 ml assayed as described before [11] using asialo-α_1-acid glycoprotein as acceptor. This enzyme presumably contained at least two distinct α1,3-fucosyltransferase activities (see "Discussion"). It was used without further purification.

Synthesis of Galα1→3Galβ1→4GlcNAc

For α1,3-galactosylation of LacNAc the following components were incubated in a final reaction volume of 1.2 ml: 120 μmol sodium cacodylic acid buffer at pH 6.5; 4.8 μmol ATP; 10 μl Triton X-100; 60 μmol $MnCl_2$; 1.2 mg bovine serum albumin; 24 μmol gamma-galactonolacton; 6 μmol N-acetyllactosamine; 7.5 μmol UDP-[^{14}C]Gal (specific radioactivity 0.1 Ci/mol), and ≈ 100 mU recombinant bovine α1,3-galactosyltransferase. The mixture was incubated for 16 h at 37°C, after which the mixture was passed through a column (4 ml bedvolume) of Dowex 1X8 (Cl^-). The run-through and washes (12 ml water) were collected and pooled, and yields were calculated from the radioactivity in the pooled product.

Prior to α1,3-fucosylation, the ^{14}C-labeled product obtained as described above was further purified by gel filtration. The product was loaded onto a calibrated column (1.6 x 200 cm)

of Bio-Gel P-4 (200-400 mesh), equilibrated and eluted in 50 mM ammonium acetate buffer at pH 5.2 and 28°C, at a flow rate of 16 ml/h. Fractions that contained ^{14}C-radioactivity and that eluted in the position of a trihexoside were pooled and lyophilized.

Part of the product (0.8 μmol) was then further purified for analysis by ^{1}H-NMR, whereas the remainder (2.5 μmol) was incubated with human milk α1,3-fucosyltransferase.

Synthesis of Galα1→3Galβ1→4[Fucα1→3]GlcNAc

The fucosylated tetrasaccharide was synthesized in a final reaction volume of 2.0 ml which contained 100 μmol sodium cacodylic acid buffer at pH 7.2; 0.2 mmol NaCl; 8 μmol ATP; 40 μmol $MnCl_2$; 1.0 ml glycerol; 1 mg sodium azide; 2.5 μmol [^{14}C]Galα1→3-Galβ1→4GlcNAc; 3.2 μmol GDP-[^{3}H]fucose (specific radioactivity 0.34 Ci/mol), and 3 mU human milk α1,3-fucosyltransferase. After incubation for 69 h at 37°C, the mixture was passed through a column (3.5 ml bedvolume) of Dowex 1X8 (Cl^--form), flow-through and washes (10 ml water) were combined, and the yield of product was calculated from the radioactivity in the pooled fractions. The product was lyophilized, and subjected to gel filtration chromatography as described above. The [^{3}H/^{14}C]-labeled material that eluted in the position of a tetrasaccharide was pooled, and processed for NMR analysis.

^{1}H-NMR analysis

Prior to ^{1}H-NMR analysis each of the enzymatic products was desalted by loading it onto a column (bedvolume 1 ml) of Dowex 50WX8 (H^+). The flow-through and washes (3 ml water) of the column were pooled, lyophilized, and the residue was dissolved into 0.5 ml of distilled water. The sample was then subjected to gel filtration chromatography on a column (10 x 45 cm) of Biogel P-2 (200-400 mesh), equilibrated and run in H_2O at a flow rate of 20 ml/h. Fractions of 1 ml were collected and radioactivity was counted. The fractions that contained radioactivity were pooled, and 300-500 nmol of product was processed for ^{1}H-NMR analysis as described previously [12]. ^{1}H-NMR spectroscopy at 400 MHz was performed on a Bruker MSL-400 spectrometer (Facility of the Department of Physics, Vrije Universiteit, Amsterdam).

HPLC characterization of the oligosaccharide products

To characterize the products formed from N-acetyllactosamine, part of the oligosaccharide materials isolated by gel filtration on Bio-Gel P-4, Dowex 1X-8 and Bio-Gel P-2 chromatography as described above, was subjected to high-pH anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) [13]. The HPAEC-PAD system consisted of a Dionex Bio-LC gradient pump, a CarboPac PA-1 column (4 x 250 mm) and a Model PAD 2 detector. The following pulse potentials and durations were used for detection: E_1 = 0.05 V (t_1 = 480 ms); E_2 = 0.60 V (t_2 = 120 ms); E_3 = -0.60 V (t_3 = 60 ms). The response time of the detector was set to 1 s. Samples were dissolved in 0.1 M NaOH and injected using a Dionex Micro Injection Valve equipped with a 25 μl sample loop. The chromatographic data were integrated and plotted using a Shimadzu C-R5A integrator. A Dionex Eluant Degas Module was employed to saturate the eluants with helium in order to degas and to minimize absorption of CO_2. For the separation of the oligosaccharides the column was eluted isocratically with 0.1 M NaOH for 10 min, after which a gradient of sodium acetate in 0.1 M NaOH was applied raising the sodium acetate concentration from 0 to 0.025 M in 10 min. The flow rate was 1 ml/min and fractions of 1 ml were collected and counted for radioactivity.

RESULTS

Synthesis of Galα1→3Galβ1→4GlcNAc and Galα1→3Galβ1→4[Fucα1→3]GlcNAc

The product obtained upon incubation of LacNAc with UDP-Gal:Galβ1→4GlcNAc α1,3-galactosyltransferase (α1,3-GT) in the presence of UDP-[^{14}C]galactose was purified by gel filtration on a calibrated column of Bio-Gel P-4. The radioactive product eluted as a single peak in the trisaccharide region. After additional purification on columns of Dowex 50W (H^+) and Bio-Gel P-2, the yield of product was calculated from the specific radioactivity and was found to be 3.3 μmol, which is 57% with respect to the limiting substrate, LacNAc.

Part of the trisaccharide, isolated as described above, was fucosylated in the next step of the synthesis. The fucosylated product was purified on a column of Bio-Gel P-4. A double-

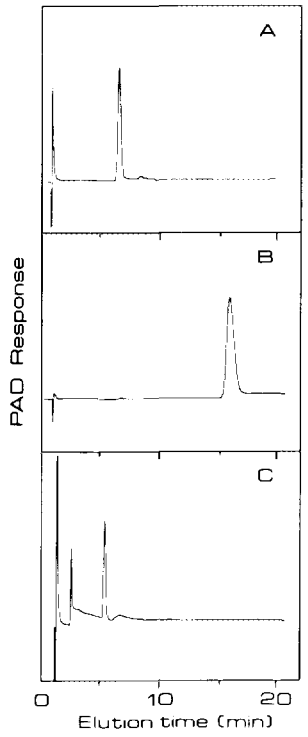

Fig. 1. Characterization by anion-exchange HPLC (HPAEC-PAD) of N-acetyllactosamine and its α1,3-galactosylated and/or α1,3-fucosylated products. Samples of 20 μl (1 nmol each) of N-acetyllactosamine and its enzymatic products were analyzed on a Dionex CarboPac PA-1 column. Elution conditions were as described under "Experimental Procedures".

labeled compound ($^{3}H/^{14}C$) eluted in the position of a tetrasaccharide. This product was pooled, and repurified as described above. The yield of product was 0.95 μmol, which is 38% with respect to the limiting substrate, [^{14}C]Galα1→3Galβ1→4GlcNAc.

Characterization of oligosaccharides by HPAEC

The retention times of LacNAc and the products formed thereof in the HPAEC system used were 6.9, 15.8 and 5.4 min for LacNAc, the trisaccharide and the tetrasaccharide product, respectively (Fig. 1). Consistent with earlier observations [11,13] addition of a fucosyl residue to an oligosaccharide results in a large reduction in retention time.

Identification of the products of α-galactosylation and α-fucosylation by 400-MHz ^{1}H NMR spectroscopy

The NMR spectral data of LacNAc and its α-galactosylated and α-fucosylated derivatives are given in Figs. 2 and 3, and Table 1. The chemical shift values and coupling constants of α-galactosylated LacNAc are essentially identical to those published before for Galα1→3-Galβ1→4GlcNAc (3'-galactosylLacNAc) [14]. In short, attachment of Gal in α1→3-linkage is revealed by the strong downfield shifts of the resonances of H-1 ($\Delta \approx +0.07$ ppm) and H-4 ($\Delta \approx +0.25$ ppm) of Galβ4 and the appearance of a doublet at 5.146 ppm of H-1 Galα3 and signals at 4.021 and 4.197 of H-4 and H-5 of this residue.

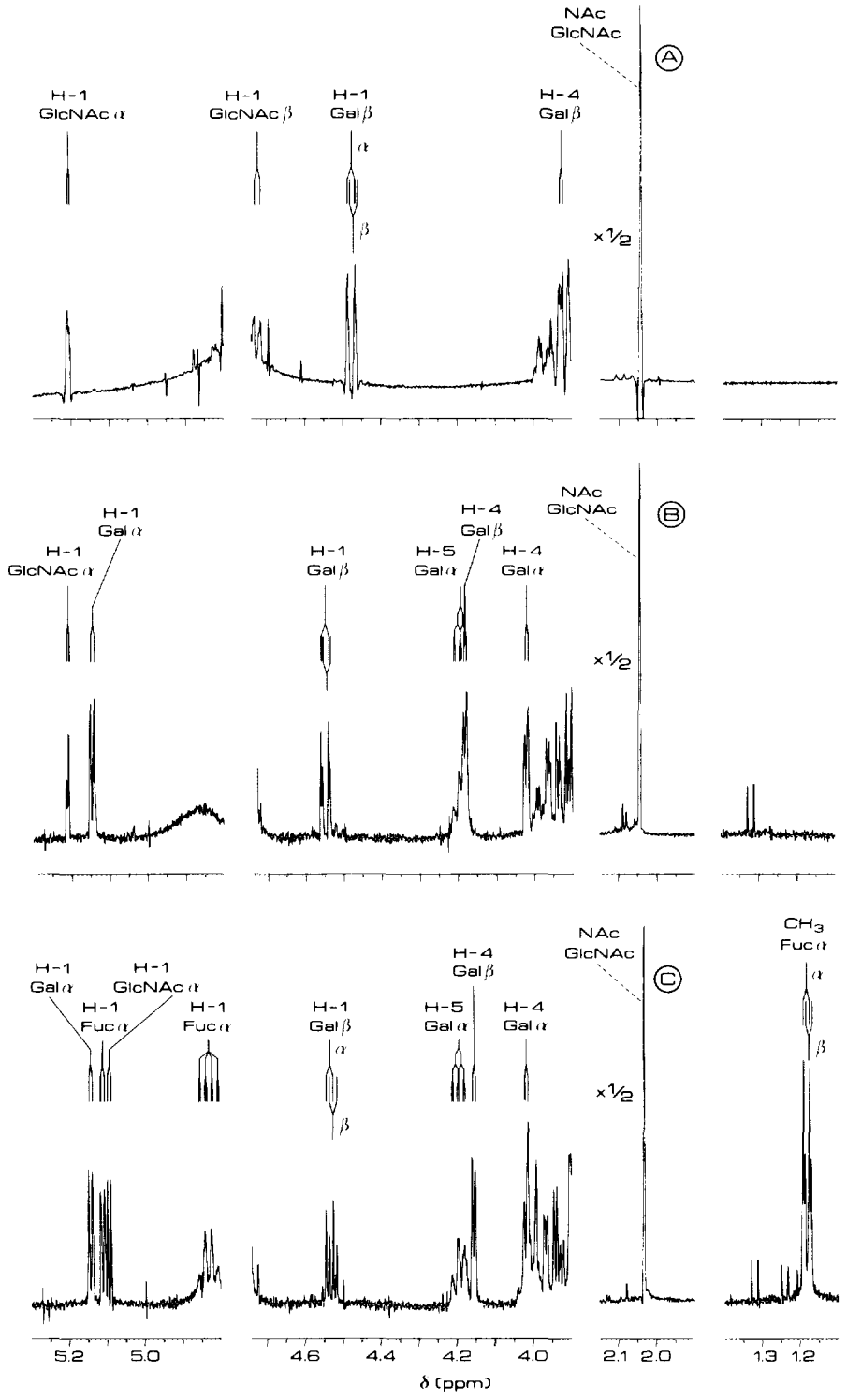

Fig. 2. 400-MHz ^{1}H NMR spectra that compare the structural reporter-group regions of the substrate Galβ1→4GlcNAc (A) with those of the product of incubation with α1,3-galactosyltransferase (B), and with those of the product obtained by incubation of Galα1→3Galβ1→4GlcNAc with human milk α1,3-fucosyltransferase (C).

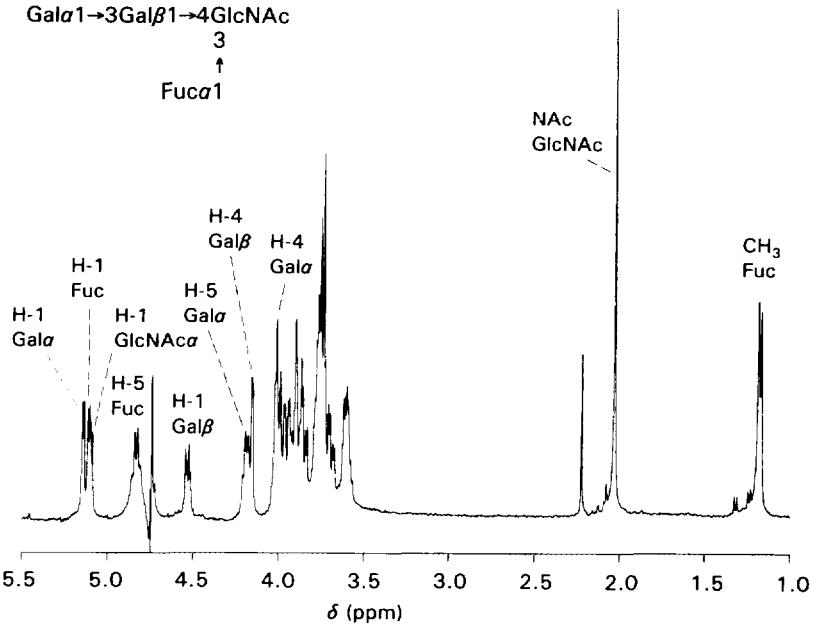

Fig. 3. 400-MHz ^{1}H NMR spectrum of the tetrasaccharide Galα1→3Galβ1→4-[Fucα1→3]GlcNAc.

The effects on the chemical shift values of subsequent fucose addition to the trisaccharide intermediate are analogous to those seen upon α1,3-fucosylation of NeuAcα2→3Galβ1→4-GlcNAc (3'-sialylLacNAc) to form the sialyl-Le^x structure (NeuAcα2→3Galβ1→4[Fucα1→3]-GlcNAc) [15-17]. Most prominent is the strong upfield shift of the resonances for H-1 GlcNAcα (Δ ≈ -0.12 ppm) which indicates that the fucose residue is in the proximity of the anomeric center. This effect is of comparable magnitude as that observed for the conversion of 3'-sialylLacNAc to sialyl-Le^x [15,17]. Interestingly the effect on H-1 GlcNAcβ is negligible. In addition, fucose attachment to 3'-galactosylLacNAc causes upfield shifts of the resonances of H-1 Galβ (Δ ≈ -0.02 ppm), H-4 Galβ (Δ ≈ -0.03 ppm) and NAc GlcNAc (Δ ≈ -0.01 ppm). A similar effect is produced by α1,3-fucosylation of 3'-sialylLacNAc. By contrast small effects are observed on the resonances of the H-1, H-4 and H-5 protons of Galα indicating that the α-galactosyl residue is remote from the fucosyl residue. Furthermore, fucose addition brings about the appearance of a set of new resonances representing the H-1 (δ = 5.111 and 5.108 ppm), H-5 (δ = 4.196 ppm) and CH_3 (δ = 1.185 and 1.180 ppm) protons of the added residue. These resonances are similar yet not identical to those in the spectrum of the sialyl-Le^x tetrasaccharide [15,16].

Based on these spectral data along with the known linkage specificity of the α1,3-galactosyltransferase [12] and the α1,3-fucosyltransferase [9] used, the chromatographic behaviour of the product upon HPAEC, and the 1:1 molar ratios of residues calculated from the ^{3}H- and ^{14}C-radioactivity, it is concluded that the structures of the trisaccharide and the tetrasaccharide products are Galα1→3Galβ1→4GlcNAc and Galα1→3Galβ1→4[Fucα1→3]-GlcNAc, respectively.

DISCUSSION

In this report we describe the enzymatic synthesis of α1,3-galactosylated, α1,3-fucosylated N-acetyllactosamine. Successive incubations of LacNAc with recombinant α1,3-

Table 1. 400-MHz ^{1}H NMR chemical shift values (ppm) and J coupling constants (Hz) of structural reporter-group protons of the constituent monosaccharides of N-acetyllactosamine, and its α1,3-galactosylated and α1,3-galactosylated/α1,3-fucosylated derivative

Reporter group	Residue	Anomer of compound	Chemical shift (coupling constant)[a] for		
			Galβ1→4GlcNAc	Galα1→3Galβ1→4GlcNAc	Galα1→3Galβ1→4GlcNAc (3 ↑ Fuc1α)
			ppm (Hz)		
H-1	GlcNAc	α	5.207 (2.1)	5.210 (2.5)	5.094 (3.4)
		β	~4.724	~4.728	~4.731
	Galβ4	α	4.478 (7.8)	4.550 (7.8)	4.535 (7.8)
		β	4.475 (7.8)	4.546 (7.8)	4.526 (7.7)
	Galα3	α	—	5.146 (3.9)	5.143 (3.9)
		β			
	Fucα3	α	—	—	5.111 (4.1)
		β	—	—	5.108 (4.1)
H-4	Galβ4	α	3.928 (3.3)	4.182 (3.0)	4.156 (3.3)
		β			
	Galα3	α	—	4.021 (3.3)	4.019 (3.6)
		β			
H-5	Galα3	α	—	4.197 (6.3)	4.196 (6.5)
		β			
	Fucα3	α	—	—	4.835 (6.6)
		β			
CH_3	Fucα3	α	—	—	1.185 (6.6)
		β	—	—	1.180 (6.6)
NAc	GlcNAc	α	2.043	2.044	2.033
		β			

[a]Coupling constants are for H-1 $J_{1,2}$; for H-4 $J_{3,4}$; for H-5 and CH_3 $J_{5,6}$.

galactosyltransferase and human milk α1,3-fucosyltransferase generated micromol amounts of a tetrasaccharide product. ^{1}H-NMR analysis, and analysis of the behaviour of the product in anion-exchange HPLC and gel filtration chromatography confirmed that stepwise addition of one α-Gal and one α-fucose residue produced the compound Galα1→3Galβ1→4[Fucα1→3]-GlcNAc.

The order of addition of the sugar residues is not random: It has been shown previously that the Le^x structure is not a substrate for bovine α1,3-GT [4]. The presence of fucose in the acceptor, either α1,2-linked to the terminal Gal, or α1,3-linked to the subterminal GlcNAc, effectively blocks α1,3-galactosylation. In contrast, α1,3-fucosylation of the trisaccharide Galα1→3Galβ1→4GlcNAc is a relatively efficient reaction. Therefore, production of the Galα1→3-Le^x structure *in vivo* would require that α1,3-galactosylation takes place prior to α1,3-fucosylation (cf. the pathway presented in Fig. 4). Analogously, synthesis of the sialyl-Le^x and the Le^y determinant requires that α2,3-sialylation and α1,2-fucosylation, respectively, precedes α1,3-fucosylation [18,19].

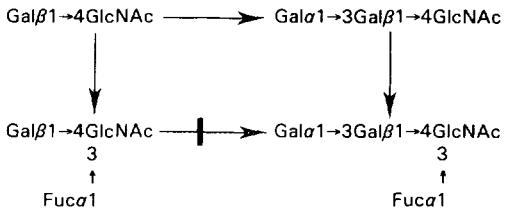

Fig. 4. Preferred ordered pathway for the synthesis of the tetrasaccharide Galα1→3Galβ1→4[Fucα1→3]GlcNAc. Based on the known acceptor substrate specificities of bovine α1,3-galactosyltransferase and human milk α1,3-fucosyltransferase an ordered pathway is proposed for the enzymatic synthesis of the Galα1→3-Le^x determinant.

Which α1,3-fucosyltransferase is involved in the production of the tetrasaccharide ? Human milk contains at least two N-acetylglucosaminide α1,3-fucosyltransferase activities: The Lewis gene-encoded fucosyltransferase III, and an enzyme activity similar to fucosyltransferase V or VI [9, 20-22]. Partially purified human milk enzyme acts both on type 1 and type 2 acceptors: Significant activities are obtained on the one hand with lacto-N-biose I (Galβ1→3GlcNAc), and on the other with LacNAc and various 2'- and 3'-substituted derivatives thereof [9, 20-22], and also with 2'-fucosyllactose [9,22]. Apparently the human milk enzyme acts on LacNAc regardless of whether the galactosyl residue is substituted at C-2 or C-3 or not. Therefore the activity with 3'-galactosylLacNAc as demonstrated in this study is not unexpected.

The presence of fucosyltransferase III should account for the transfer to type 1 acceptors. Cloned fucosyltransferase III (Lewis) acts on lacto-N-biose I, but is much less active with LacNAc and 3'-sialylLacNAc [23]. It is likely that the activity with type 2 acceptors is due to either fucosyltransferase V or -VI, or both, based on reported acceptor properties of recombinant enzymes [23-25]. Both cloned fucosyltransferase V and VI acts on LacNAc, 3'-sialylLacNAc and lactose, but little or no activity is observed when lacto-N-biose I is used as the acceptor [23-25]. Taken together these data suggest that most likely the fucosyltransferase V and/or -VI activity present in human milk is responsible for the synthesis of Galα1→3-Le^x as described in this paper.

Fucosyltransferases with a specificity similar to that of fucosyltransferase V seem to occur in a variety of mammalian tissues [21,26]. In view of the ease and efficiency of *in vitro* enzymatic synthesis, it seems surprising that the occurrence of the Galα1→3-Le^x structure in N- or O-glycoproteins has not yet been reported. This may be due to the fact that it is only a minor constituent of glycoproteins, or that it is present in only a limited subset of cells and tissues. Alternatively, the fact that the synthetic pathway involves an ordered stepwise addition of sugar residues may limit the occurrence of the determinant *in vivo*. Compartmentalization of the glycosyltransferases, in combination with a high degree of acceptor specificity, could prevent the synthesis of Galα1→3-Le^x in mammalian cells. Whether the structure is generated *in vivo* is likely to depend on the substrate specificity of the various α1,3-fucosyltransferases, and their subcellular localization with respect to the α1,3-galactosyltransferase. As a terminal glycosyltransferase, α1,3-GT may be located in a distal compartment, possibly the *trans*-Golgi. There are at least five different fucosyltransferases [23,24,26], the localization of which is unknown as yet. Some of these enzymes may act before α1,3-GT does, and in this way block the synthesis of the Galα1→3-Le^x structure (Fig. 4).

Over the last few years a variety of fucosylated carbohydrate structures have been implicated in cell adhesion processes. In particular the sialyl-Lewis^x determinant (NeuAcα2→3Galβ1→4[Fucα1→3]GlcNAc has been shown to be involved in tumor cell metastasis and leukocyte trafficking, by mediating interactions between endothelial cells and lymphocytes and granulocytes [27]. Related compounds such as the sialyl-Le^a and the sialylated, fucosylated VIM-2 determinant, but also sulphated structures analogous to the sialyl-Le^x structure may play a role in the same recognition system [27-29]. All these determinants are recognized by carbohydrate-binding receptor proteins of the "selectin" family [1,2,27]. In contrast, the non-sialylated trisaccharide structure Le^x (CD15) seems to be less effective.

Apparently, specific modification of a common lactosaminoglycan "backbone" yields determinants that may function as ligands in cellular adhesion. These modifications may produce a novel terminal structure, or a involve a novel combination of known elements such as present in the ligand for L-selectin [3]. It will be of interest to determine whether the Galα1→3-Le^x structure, present on N- or O-linked carbohydrate chains, could be another cell adhesion determinant. With the availability of a flexible method for its synthesis this question can now be experimentally approached.

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