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In vitro α1-3 or α1-4 fucosylation of type I and II oligosaccharides with secreted forms of recombinant human fucosyltransferases III and VI

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Transgalactosylation of chitobiose and chitotriose employing β-galactosidase from bovine testes yielded mixtures with β1-3 linked galactose (type I) and β1-4 linked galactose (type II) in a final ratio of 1:1 for the tri- and 1:1.4 for the tetrasaccharide. After 24 h incubations of the two purified oligosaccharide mixtures with large amounts (20-fold increase compared with standard conditions) of human α1, 3/4-fucosyltransferase III (FucT III), the type I tri-/tetrasaccharides were completely converted to the Lewis^a structure, whereas approximately 10% fucosylation of the type II isomers to the Lewis^x oligosaccharides was observed in long-term incubations.

Employing large amounts of human α1, 3-fucosyltransferase VI (FucT VI), the type I trisaccharide substrate was exclusively fucosylated at the proximal O-4 substituted N-acetylglucosamine (GlcNAc) (20%) whereas almost all of the type II isomers was converted to the corresponding Lewis^x product. 45% of the type I tetrasaccharide was fucosylated at the second GlcNAc solely by FucT VI. The type II isomer was almost completely α1-3 fucosylated to yield the Lewis^x derivative with traces of a structure that contained an additional fucose at the reducing GlcNAc. The results obtained in the present study employing high amounts of enzyme confirmed our previous results that FucT III acts preponderantly as a α1-4 fucosyltransferase onto GlcNAc *in vitro*. Human FucT VI attaches fucose exclusively in an α1-3 linkage to 4-substituted GlcNAc *in vitro* and does not modify any 3-substituted GlcNAc to yield Lewis^a oligosaccharides. With 8-methoxycarbonyloctyl glycoside acceptors used under standard conditions, FucT III acts exclusively on the type I and FucT VI only on the type II derivative. With lacto-*N*-tetraose, lacto-*N*-fucopentraose I, or LS-tetrasaccharide as substrates, FucT III modified the 3-substituted GlcNAc and the reducing glucose; FucT VI recognized only lacto-*N*-neotetraose as a substrate.

Keywords: **Fucosyltransferase, Lewis oligosaccharides, mass spectrometry**

Abbreviations: **dHex, deoxyhexose; CID, collision-induced dissociation; COSY, correlated spectroscopic DNA, deoxyribonucleic acid; ESI, electrospray ionisation mass spectrometry; FucT III, α1,¾-fucosyltransferase III, FucT VI, α1,3-fucosyltransferase VI; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Hex, hexose; HexNAc, *N*-acetylhexosamine; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; Le^a, Lewis^a; Le^x, Lewis^x; LNFP-I, lacto-*N*-fucopentaose I; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; LST-a, LS-tetrasaccharide a; ROESY, rotating frame Overhauser enhancement spectroscopy; SLN, 3′-sialyl-*N*-acetyllactosamine; sLe^a, sialyl-Lewis^a, sLe^x, sialyl-Lewis^x; TOCSY, total correlated spectroscopy**

Introduction

Lewis^x (Le^x) and Lewis^a (Le^a) type structures, as well as their sialylated or sulfated derivatives on polypeptides or glycolipids, are ligands for the selectins (P-, L-, and E-selectin) [1, 2] and are found on the cell surface of, e.g., leukocytes and tumor cells. The Le^a structures are frequently detected in high amounts on cancer cells of the digestive tract [3]. The involvement of Lewis type glycoconjugates in recognition processes during inflammation, lymphocyte migration, and metastasis mediated by selectins opens a way to the therapeutic inhibition of the selectins in such pathological processes by administration of small, chemically, or enzymo-chemically synthesized carbohydrate analogs [4], which are expected to have low immuno-

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genic properties. For this, knowledge of the substrate specificity of the eight different fucosyltranferases currently known is indispensible. In the literature, different substrate specificities for human FucT III have been reported [5–9, 21,22].

In many cases, the use of extremely high substrate concentrations, or different amounts of enzyme as well as different enzyme sources, may account for the conflicting results published in the literature [7, 8]. In the case of recombinantly expressed transferase constructs, encoding enzyme variants with different N-terminal deletions or fusion of the N-terminus of the enzyme to peptide tags or polypeptide sequences may explain the observed variations. The expression of secreted forms of recombinant human glycosyltransferases offers the opportunity of producing enzyme in large quantities which cannot be obtained from natural sources and at the same time avoids contamination of preparations with other transferases of different substrate specifity.

In the present study, the *in vitro* formation of Le^a or Le^x type oligosaccharides from a mixture of *in vitro* β1-3 and β1-4 galactosylated chitobiose or chitotriose was investigated, employing recombinant secretory forms of human FucT III [8] and FucT VI [10]. The two fucosyltransferases were compared for their activity with small oligosaccharides as well as biantennary asialo or α2-3 sialylated *N*-glycans. Products were characterized by complementary techniques, using tandem mass spectrometry, methylation analysis, high performance anion exchange chromatograph with pulsed amperometric detection (HPAEC-PAD), and 1D and 2D NMR experiments.

Materials and methods

Materials

The Gal(β1-3)GlcNAc-O$(CH_2)_8$COOMe and Gal(β1-4)GlcNAc-O$(CH_2)_8$COOMe acceptors were a gift from Dr. O. Hindsgaul (University of Alberta, Canada). GDP-fucose was purchased from Sigma. GDP-[^{14}C]-fucose (285 mCi/mmol) was from Amersham (Braunschweig, FRG). Lacto-*N*-tetraose (LNT, Galβ1-3GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-neotetraose (LNnT, Galβ1-4GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-fucopentaose I (LNFP-I, Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc), LS-tetrasaccharide a (LST-a, NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc), and 3′-sialyl-N-acetyllactosamine (SLN, NeuAcα2-3Galβ1-4GlcNAc) were bought from Oxford Glycosystems, United Kingdom. *N*-linked complex-type biantennary structures with no, one, or two α2,3-linked NeuAc residues were isolated from recombinant glycoproteins expressed from BHK-21 and CHO cells and were structurally characterized by NMR and mass spectrometric techniques as described previously [11–13].

Synthesis of GDP-Fractogel

Fractogel EMD epoxy (Merck, Darmstadt, FRG) was converted with phosphoric acid to a gel containing free phosphate groups. The gel was loaded with the nucleotide by reaction with guanosine-monophosphomorphilidate-4-morpholine-N,N′-dicyclohexylcarboxaminidine salt [25], achieving a concentration of bound nucleotides in the range of 5–30 μmol per ml of wet gel. This gel was used for the purification of both recombinant fucosyltransferases as described in Johnson *et al.* [8].

Expression of secretory forms of human fucosyltransferases III and VI in BHK-21 cells

A secretory form of human FucT III (s2FT3T2) was purified from the supernatant of transfected BHK-21 cells as described previously [8]. A secretory form of human FucT VI was generated by replacing the first 51 *N*-terminal amino acids with the human IL-2 signal sequence by PCR-mediated, site-directed mutagenesis of human genomic DNA isolated from the human glioma cell line A172 and was cloned into the eukaryotic expression vector pCR3 using a TA cloning kit (Invitrogen, Leek, the Netherlands). Transfection of BHK-21 cells with the vector pCRs2FT6 and isolation of stable cells was performed as described [8, 10]. Serum-free cell supernatants from confluent monolayers grown in 175cm^2 flasks were harvested every two days and were stored frozen until purification by affinity chromatography.

Fucosyltransferase activity determinations

Fucosyltransferase activity was determined using 8-methoxycarbonyloctyl glycoside acceptors type I and II and was tested as described previously [8], except that Mes/KOH buffer pH 6.2 was used instead of Mops/NaOH. 8-Methoxycarbonyloctyl glycoside acceptors were used at 0.3 mM concentrations and 0.5 mM GDP-fucose containing 10,000 cpm GDP-[^{14}C]-fucose/nmol. SLN, LnNT, LNT, and LST-a were used at a 0.33 mM concentration in the presence of 0.42 mM GDP-Fuc, employing either 2 μl of a FucT III preparation that formed 2 nmol/h of product with the type I 8-methoxycarbonyloctyl glycoside acceptor or 4 μl of a FucT VI that formed 2 nmol/h of product with the type II 8-methoxycarbonyloctyl glycoside acceptor. Reducing *N*-glycans of the biantennary complex type with no, one, or two α2-3 linked NeuAc were used at 0.1 mM to 0.2 mM concentrations in the presence of 0.40 mM GDP-Fuc for 4 to 24 hours. Aliquots of the reaction mixtures with oligosaccharides and N-glycans as acceptors were analyzed by HPAEC-PAD with or without desialylation with *Vibrio cholerae* sialidase as described [8]. The reaction mixtures with 8-methoxycarbonyloctyl glycoside as acceptors were diluted with water to 1 ml and applied on Sep-Pak C_{18} cartridges which were washed with 5 ml of water. The products were

eluted with 1 ml methanol. The incorporation of [^{14}C]-fucose was determined by liquid scintillation counting.

Transgalactosylation with bovine testes galactosidase

The chito-oligomers (15 mmol) were incubated at pH 4.3 (sodium phosphate-citrate buffer) with p-nitrophenyl β-D-galactopyranoside (25 mmoles) and β-galactosidase from bovine testes (160 units/1 mmol p-nitrophenyl-β-D-galactopyranoside) at 37°C for 48 h as described [14].

Incubations of Galβ1-3/4(GlcNAc)$_2$ or Galβ1-3/4(GlcNAc)$_3$ mixtures with FucT III and VI

For comparison of the activities of fucosyltransferase III and VI with the O-3/4-galactosylated GlcNAc$_2$ or GlcNAc$_3$ mixtures, 20- to 40-fold higher enzyme concentrations than previously were used [8]. Galβ1-3/4(GlcNAc)$_2$ or Galβ1-3/4(GlcNAc)$_3$ mixtures were used at 1.7 mM concentrations in the presence of 3.4 mM GDP-fucose for 24 h at 37°C. Aliquots corresponding to 3–5 nmol of substrate were withdrawn after 2, 4, and 24 h. Protein was precipitated with 5 volumes of −20°C ethanol and the dried supernatant was dissolved in H_2O, desalted, and subsequently analyzed by HPAEC-PAD. Conversion to product was detected by the appearance of a new peak with lower retention time and was quantitated by integration.

Commercial low molecular weight oligosaccharides as substrates for recombinant human FucT III and VI

The two recombinant fucosyltransferases were tested for their activity with LNFP-I, LNT, LST-a, LNnT, and SLN under identical conditions. Each enzyme was applied in amounts that led to fucosylation of 2 nmol of 8-methoxycarbonyloctyl glycoside acceptor (type I with FucT III and type II with FucT VI) within 1 h of incubation as described under Materials and Methods. The values of incorporated fucose (compare Costa, *et al.*, 1997) were calculated from peak areas in HPAEC-PAD. With FucT III about 20% to 50% of the LNT, LNFP-I, and LST-a was fucosylated after 4 h of incubation, whereas FucT VI acted only on LNnT (8%) and to a small extent with SLN, detected only after fivefold longer incubation times (Table 3).

Fucosylation of biantennary type II N-glycans

Complex-type biantennary asialo and mono- or disialylated (NeuAc α2-3-linked) *N*-glycans with proximal fucose were isolated and characterized as described previously [11–13] and were incubated with the recombinant fucosyltransferases as described above for the commercial milk oligosaccharides. *N*-glycan concentrations were 80 nmoles/ml using incubation times of up to 24 h. No degradation of the complex type oligosaccharides occurred during assay conditions.

Separation of oligosaccharides by HPLC on NH_2-bonded phase

HPLC on NH_2-Phase (Lichrosorb-NH_2, 0.4 cm × 25 cm, 5 μm particle size, Merck, Darmstadt, FRG) was performed at a flow rate of 1 mL/min. A 10 min isocratic run with 100% solvent A was followed by a linear 140 min gradient to 100% solvent B: A = 100% acetonitrile; B = 80% acetonitrile in water. Carbohydrates were detected by absorption at 206 nm.

High-pH anion-exchange chromatography of oligosaccharides

A Dionex BioLC System (Dionex, Sunnyvale, California) equipped with a CarboPac PA1 column was used in combination with a pulsed amperometric Detector (Detector Potentials E - 1, E - 2, E - 3 were +0.05, +0,60 and −0.60 V; t − 1 = 480ms, t − 2 = 120ms, t − 3 = 60ms); output range = 300nA. Elution of oligosaccharides was performed using a 40 min linear gradient from 100% solvent C to 20% solvent D followed by a 10 min rise to 100% solvent D. C: 0.1M NaOH; D: 0.6 M NaOAc in 0.1 M NaOH.

Methylation analysis

For methylation analysis and ESI-MS/MS, oligosaccharides were permethylated according to Hakomori [15], purified, hydrolyzed, reduced, and peracetylated as described [11]. Separation and identification of partially methylated alditol acetates were performed on a Finnigan gas chromatograph (Finnigan MAT corp., San Jose, California), equipped with a 30-meter DB5 capillary column and connected to a Finnigan GCQ ion trap mass spectrometer.

Electrospray ionisation tandem mass spectrometry (ESI-MS/MS)

A Finnigan MAT TSQ 700 triple quadrupole mass spectrometer equipped with a Finnigan electrospray ion source (Finnigan MAT corp., San Jose, California) was used for ESI-MS. The reduced and permethylated samples were dissolved in acetonitrile, saturated with NaCl (concentrations approximately 10 pmol/μL), and injected at a flow rate of 1 μL/min into the electrospray chamber. In the positive-ion mode, a voltage of +5.5 kV was applied to the electrospray needle. For collision-induced dissociation (CID) experiments, parent ions were selectively transmitted by the first mass analyzer and directed into the collision cell (argon was used as collision gas) with a kinetic energy set around minus 60 eV.

600-MHz ^{1}H NMR Spectroscopy

Prior to ^{1}H NMR spectroscopic analysis, the carbohydrates were repeatedly lyophilized against D_2O (Fluka, >99.95 atom% D) at pD 7 and ambient temperature. ^{1}H NMR spectra at 600 MHz were recorded at 300 K on a

Bruker AVANCE DMX 600 NMR spectrometer incorporating a gradient unit. A 1.3 s presaturation pulse was employed prior to the experimental pulse sequence to suppress the signal of the residual HOD resonance. Chemical shifts are expressed in ppm downfield from the signal for internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured by reference to free acetate (δ 1.908 in D_2O at pD 6–8 and 27°C), with an accuracy of 0.002 ppm. ^1H 2D correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), and rotating frame Overhauser enhancement spectroscopy (ROESY) were performed on the same instrument with mixing times of 80 and 500 ms, respectively, for the two latter techniques. All 1D and 2D spectra were recorded using modified Bruker software.

Results

Transgalactosylation of chito-biose and -triose

Di-*N*-acetylchitobiose (**A**) and Tri-*N*-acetylchitotriose (**B**) were transgalactosylated using the procedure described in Materials and Methods with an approximate yield of 26% in both cases. The products were isolated by Biogel P2 chromatography. Analysis of the purified oligosaccharide material by HPAEC-PAD indicated the presence of a mixture of two isomers in a ratio of approximately 1:1 for the product from chitobiose (**A.1**, **A.2**, Figure 1A) and 1:1.4 for the product from chitotriose as starting material (**B.1**, **B.2**, Figure 1D). After isolation of approximately 10 µg of each compound in a preparative HPAEC run, their structures were elucidated after reduction and permethylation by

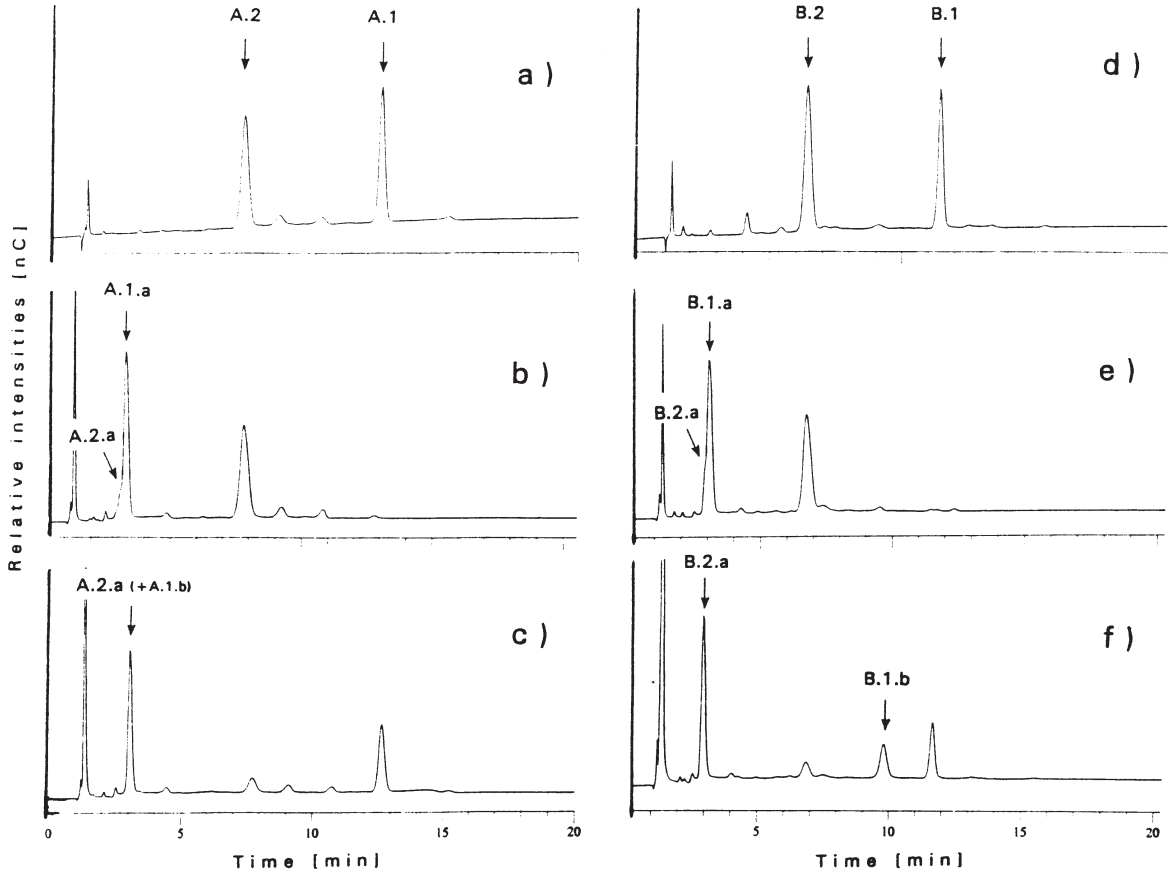

Figure 1. HPAEC-PAD analyses of the transgalactosylation products of chitobiose (a–c) and chitotriose (d–f).
A) Before incubation with FucT III or FucT VI [**A.2** = Galβ1-4GlcNAcβ1-4GlcNAc; **A.1** = Galβ1-3GlcNAcβ1-4GlcNAc].
B) After incubation with FucT III. **A.1** is completely converted to the Le^a containing product **A.1.a** [Galβ1-3(Fucα1-4)GlcNAcβ1-4GlcNAc], whereas some 10% of **A.2** is converted to the isomeric Le^x structure **A.2.a** (peak shoulder **A.2a**).
C) After incubation with FucT VI, **A.2** is almost completely converted to the corresponding Le^x product **A.2.a** [Galβ1-4(Fucα1-3)GlcNAcβ1-4GlcNAc], whereas some 20% of **A.1** are fucosylated at the proximal GlcNAc to yield **A.1.b** (not separated from **A.2.a**).
D) Before incubation with FucT III or FucT VI [**B.2** = Galβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAc; **B.1** = Galβ1-3GlcNAcβ1-4GlcNAcβ1-4GlcNAc].
E) After incubation with FucT III, **B.1** is completely converted to the Le^a containing product **B.1.a** [Galβ1-3(Fucα1-4)GlcNAcβ1-4GLCNAcβ1-4GlcNAc], which is barely separated from its Le^x isomer **B.2.a** (ca. 10% conversion).
F) After incubation with FucT VI, **B.2** is almost completely converted to the Le^x containing product **B.2.a** [Galβ1-4(Fucα1-3)GlcNAcβ1-4GlcNAcβ-1-4GlcNAc]. Approximately 45% of **B.1** is fucosylated at O-3 of the inner GlcNAc residue to yield **B.1.b** [Galβ1-3GlcNAcβ1-4(Fucα1-3)GlcNAcβ1-4GlcNAc]

Table 1. Methylation analyses. Compounds are numbered as in the text and Figure 5. The compounds marked by an asterisk were analyzed twice with identical results after separation by preparative HPAEC/PAD or aminophase HPLC. FT6-A and FT6-B are from the desalted total incubation mixtures of FucT VI with either beta1-3/4-galactosylated chito**bi**ose (**A.1***;* **A.2**) or chito**tri**ose (**B.1***;* **B.2**)

Peracetylated derivative of	*A.1*	*A.2**	*B.1*	*B.2**	*A.1.a*	*B.1.a*	*FT6-A*	*FT6-B*
Fucitol								
2,3,4-Tri-O-methyl-Galacitol	—	—	—	—	1.0	0.9	0.7	0.7
2,3,4,6-Tetra-O-methyl-2-N-Methylacetamido-2-deoxyglucitol	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1,3,5,6-Tetra-O-methyl-	1.0	1.0	1.0	1.0	1.0	1.0	0.8	1.0
1,5,6-Tri-O-methyl-	—	—	—	—	—	—	0.2	<0.1
3,6-Di-O-methyl-	—	0.9	1.0	2.0	—	0.9	0.1	1.0
4,6-Di-O-methyl-	0.6	—	1.0	—	—	—	0.4	0.3
6-O-methyl-	—	—	—	—	1.0	1.1	0.5	

methylation analysis (Table 1) and ESI-MS/MS (Figure 2). Compounds **A.1** and **B.1** yielded the 2-*N*-acetylaminoglucitol derivative indicative of a GlcNAc residue monosubstituted at O-3, implying a 1-3 galactosylation of both chitooligosaccharides at the terminal GlcNAc residue, whereas compounds **A.2** and **B.2** yielded, in contrast, only the derivative characteristic for a four-substituted subterminal GlcNAc residue, indicating a 1-4-galactosylation of both oligosaccharides. These results were confirmed by ESI-MS/MS of the reduced and permethylated compounds. As expected, the isomeric oligosaccharides **A.1** and **A.2**, as well as **B.1** and **B.2**, yielded identical molecular ions (sodium adducts) at m/z 779 [Hex HexNAc HexNAc-ol+Na] and 1024 [Hex $HexNAc_2$ HexNAc-ol+Na], respectively. On CID of the molecular ions, the pairs of isomers generated clearly different daughter ion spectra, allowing a clear distinction between the 3- and 4-galactosylated species (Figure 2). The well-known [16] preferential elimination of the galactose linked to O-3 of a GlcNAc-R moiety compared with the four-linked isomer led to the observation of intense fragments ions at m/z 259, corresponding to a terminal hexose residue and the complementary fragment at m/z 543 [M-Hex] in the case of the type I structural motif (compounds **A.1.** and **B.1**; Figure 2A), whereas these fragments ions are very weak or absent in the isomeric type II compound (compounds **A.2** and **B.2**; Figure 2B). (See abbreviations list for explanation of Hex and HexNAc.)

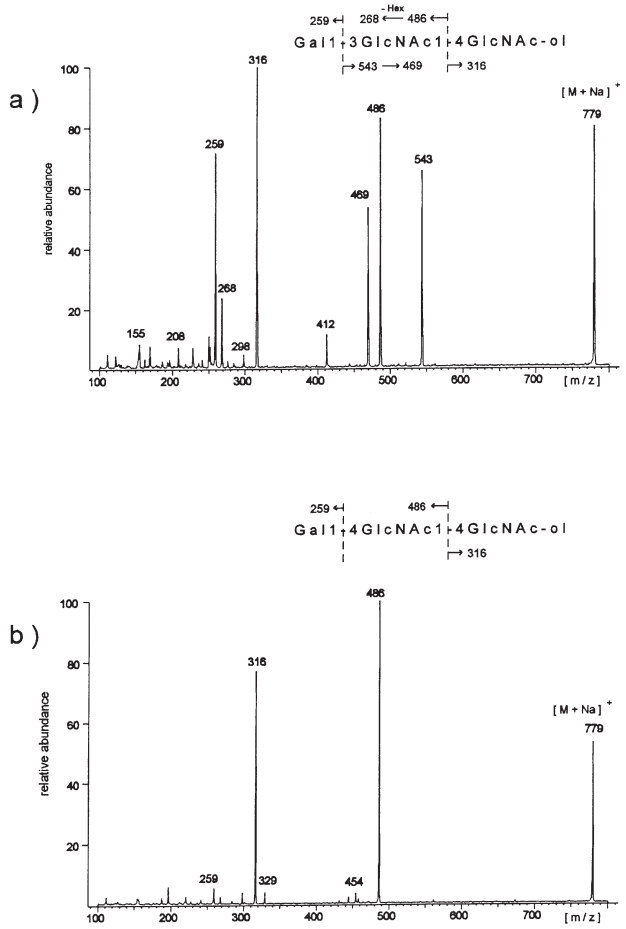

Figure 2. A) Daughter ion spectra of the reduced and permethylated sodium adducts of galactochitobiose trisaccharides **A.1** [Galβ1-**3**GlcNAc-ol]; b) **A.2** [Galβ1-**4**GlcNAc1-4GlcNAc-ol] that allow unequivocal discrimination between the two isomers. The type I compound (spectrum a) exhibits the well known preferred elimination of the three-linked substituent at the GlcNAc residue, leading to an intense fragment at m/z 543 confirmed by the complementary signal characteristic for a Hex residue at m/z 259, that is not (or with much weaker intensity) observed in the 4-substituted isomer.

Fucosylation of the galactosylated chitooligomers with recombinant human Fucosyltransferase III

The mixtures of type I and II type structures generated by the transgalactosylation procedure were separately subjected to *in vitro* fucosylation experiments with 20–40 times larger amounts of purified human FucT III than were used previously [8]. After incubation (see Materials and Methods) of the isomer mixture **A.1**+**A.2** with FucT III (Figure

5a, upper panel), the resulting oligosaccharides were analyzed by HPAEC-PAD (Figure 1B). Whereas the type II material (**A.2**) remained predominantly unchanged, the isomeric type I material (**A.1**) was almost completely converted to an earlier eluting oligosaccharide **A.1.a**. For exact structural characterization, the incubation products were separated into two fractions by aminophase HPLC. The earlier eluting peak was shown, as expected, to consist of unreacted **A.2** by methylation analysis (Table 1) and ESI-MS/MS (compare Figure 1). The structure of the later eluting peak was elucidated by 1D and 2D NMR spectrometry, methylation analysis, and ESI-MS/MS after reduction and permethylation. The 600 MHz 1D ^{1}H NMR spectrum (Table 2, Figure 3) clearly showed the presence of one dominant tetrasaccharide species: **A.1.a** bearing an α-linked fucose residue. The resonances of the anomeric proton of this fucose were in excellent agreement with published data for oligosaccharides incorporating the Le^a structural motif [17]. The presence of small amounts of an additional oligosaccharide species **A.2.a** (ca. 8% by integration) was detected from a set of weak signals that appeared in the anomeric proton region of the 1D NMR spectrum. The chemical shifts of these resonances were in good agreement with NMR data published for Le^x containing structures [18], and thus indicated the presence of small amounts of a Le^x compound bearing galactose linked to O-4 and fucose linked to O-3 of the subterminal GlcNAc residue.

The linkage positions of the galactose and fucose residues in **A.1.a** were then rigorously determined by 2D NMR techniques. Three pyranose monosaccharide spin systems, two with β-anomeric configurations and one with an α-configuration, and the α- and β-anomers of the reducing proximal GlcNAc residue, also a pyranose, were completely or partially assigned using 2D COSY and TOCSY spectra (Table 2). Interresidual cross-peaks in the 2D ROESY spectrum were then used to determine the linkages between the monosaccharide residues. Thus, a cross-peak on the H-1 trace of the galactose residue corresponding to the H-3 proton of the subterminal GlcNAc residue and a signal corresponding to H-4 of the same residue on the H-1 trace of the fucose residue indicated the 1–3 and 1–4-linkages between these residues unequivocally demonstrating the presence of the Le^a structural motif. The respective 2D NMR signals of the second component **A.2.a** present in much lower amounts were too weak to allow an unequivocal confirmation of the assumed Le^x arrangement.

Methylation analysis data were compatible with the NMR data. The presence of small amounts of a Le^x-type structure, however, could not be confirmed by this technique, since both possible Lewis isomers yield the same 3,4-disubstituted GlcNAc derivative. The partial fucosylation of the reducing GlcNAc, however, could be excluded as only the derivative characteristic of a 4-monosubstituted reducing GlcNAc was detected (see Table 1). Positive mode ESI-MS of the reduced and permethylated compound yielded a molecular ion at m/z 953 [Hex dHex HexNAc HexNAc-ol+Na], whose daughter ion spectrum generated by CID was in complete agreement with the presence of a Le^a type structure as the main component (compare Figure 2). The absence of a fragment ion at m/z 490 [dHex-HexNAc-ol] confirmed the exclusive fucosylation of the subterminal GlcNAc residue.

The isomer mixture **B.1**+**B.2** obtained by transgalactosylation of chitotriose were fucosylated with FucT III under identical conditions (Figure 5B, upper panel). After incubation for 24 h, products were desalted and analyzed by

Table 2. Chemical shifts (ppm) of oligosaccharides **A.1.a** and **B.1.a.** Unequivocal sequence specific interresidual ROE cross-peaks are indicated (R:)

A.1.a	*B.1.a*
A B C	A B C D
Galβ1-3GlcNAcβ1-4GlcNAc	Galβ1-3GlcNAcβ1-4GlcNAcβ1-4GlcNAc
/	/
Fucα1-4	Fucα1-4
E	E
Residue A	Residue A
H-1:4.497 (R: B H-3)	H-1:4.494
H-2:3.47	
H-3:3.63	Residue B
H-4:3.89	H-1:4.601
H-5:3.58	
H-6AB:3.74	Residue C
	H-1:4.578 (Dα); 4.589 (Dβ)
Residue B	
H-1:4.608 (Cα); 4.619 (Cβ)	Residue D
H-2:3.97	H-1:5.185 (α)
H-3:4.09	H-1:4.70 (β)
H-4:3.75	
H-5:3.61	Residue E
H-6A:3.98	H-1:5.007
H-6B:3.86	H-5:4.866
	CH_3:1.174
Residue C(α)	
H-1:5.192	
H-2:3.88	
H-3:3.64	
Residue C(β)	
H-1:4.70	
H-2:3.70	
H-3:3.53	
Residue E	
H-1:5.008 (R: B H-4)	
H-2:3.81	
H-3:3.89	
H-4:3.80	
H-5:4,870	
Ch_3:1.176	

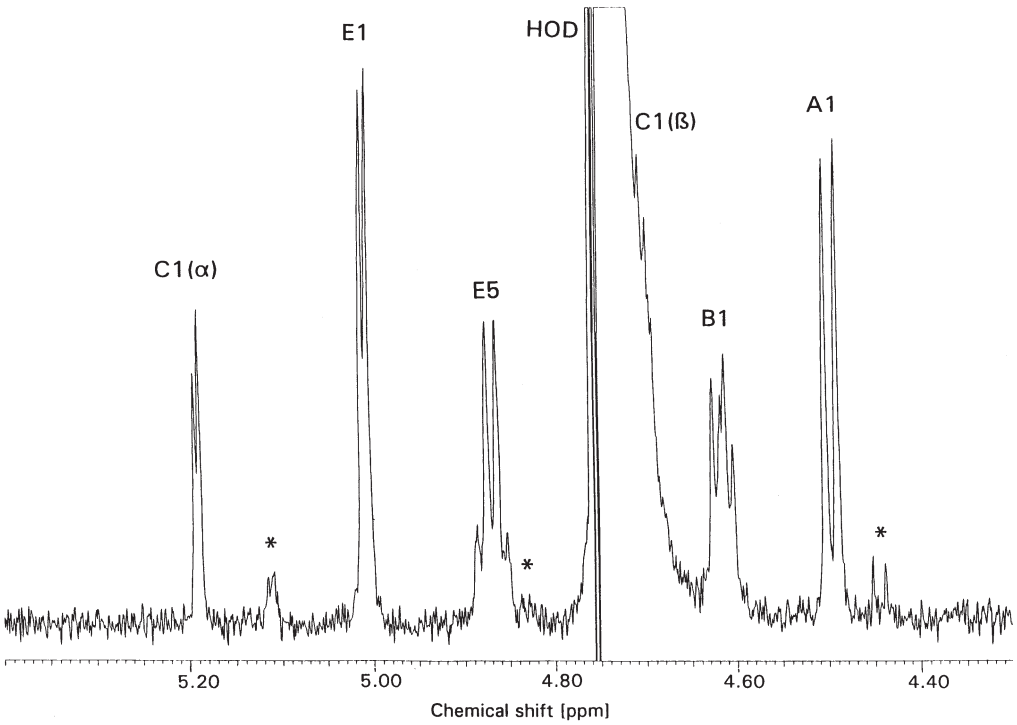

Figure 3. Anomeric proton region of the 600 MHz 1D^{1}H NMR spectrum of the fucosylation product of the 1:1 mixture of β1-3 (A.1) and β1-4 galactosylated (A.2) chitobiose after incubation with human FucT III. The chemical shifts of the anomeric protons show the presence of an oligosaccharide with the Le^a motif (**A.1.a**) arising from the α1-4fucosylation of the type I isomer (**A.1**). This finding was rigourously confirmed by 2D NMR methods (see Table 2). The signals marked by asterisks are compatible with the presence of small amounts of the isomeric structure incorporating the Le^x motif (**A.2.a**).

HPAEC-PAD (Figure 1E). Results were analogous to the incubation of the Gal3/4(GlcNAc)$_2$ derivatives described above. Structure **B.2** was shown to remain largely unchanged, whereas **B.1** was completely converted to an early eluting oligosaccharide **B.1.a**. After separation by aminophase HPLC, the unfucosylated material again contained Gal exclusively in an O-4 linkage to GlcNAc, whereas the main component **B.1.a** present in the fucosylated fraction was found to consist of Le^a type material as was indicated by the ^{1}H NMR spectrum and the ESI-daughter ion spectrum of the reduced and permethylated compound. An additional set of weak signals in the ^{1}H NMR spectrum suggested the presence of small amounts of the respective Le^x isomer **B.2.a** (ca. 12%). Any other fucosylation than the subterminal GlcNAc residue could be excluded by ESI-MS/MS of the reduced and permethylated oligosaccharide material.

Fucosylation of the transgalactosylation products with recombinant human fucosyltransferase VI

Since the data published in the literature [10, 19,20,23] on the specifity of human FucT VI indicate an exclusive α1-3fucosylation of type II structures leading to the Le^x motif, we did not subject the incubation mixtures (Figure 5A and B lower panels) of the oligosaccharides **A.1**+**A.2** and **B.1**+**B.2** to HPLC separation, but characterized the reaction products directly by HPAEC-PAD, methylation analysis, and ESI-MS/MS after desalting.

Again for the recombinant human FucT VI, enzyme amounts 20- to 40-fold higher were used than for the standard fucosyltransferase activity assay with 8-methoxycarbonyloctyl type II glycoside acceptor which yielded 2 nmol of product formed within 1 h of incubation (see Materials and Methods). HPAEC-PAD of the incubation mixture (Figure 1C) revealed an approximately 80% conversion of **A.2** to the early eluting Le^x compound **A.2.a.** However, the type I material **A.1** also unexpectedly showed a 20% conversion to a fucosylated product **A.1.b**.

After reduction and permethylation of the total oligosaccharide material, ESI-MS of the oligosaccharide mixture afforded molecular ions at m/z 779 [Hex HexNAc HexNAc-ol+Na] and 953 [Hex dHex HexNAc HexNAc-ol+Na], corresponding to unreacted starting material (approximately 40% of total) and a monofucosylated reaction product (60%) (Figure 5A, lower panel). MS/MS of the former molecular ion yielded a fragmentation pattern, indicating predominantly type I linkage of the galactose residue (compare Figure 2B) and confirming a preferential fucosylation of type II structures by FucT VI. CID of the

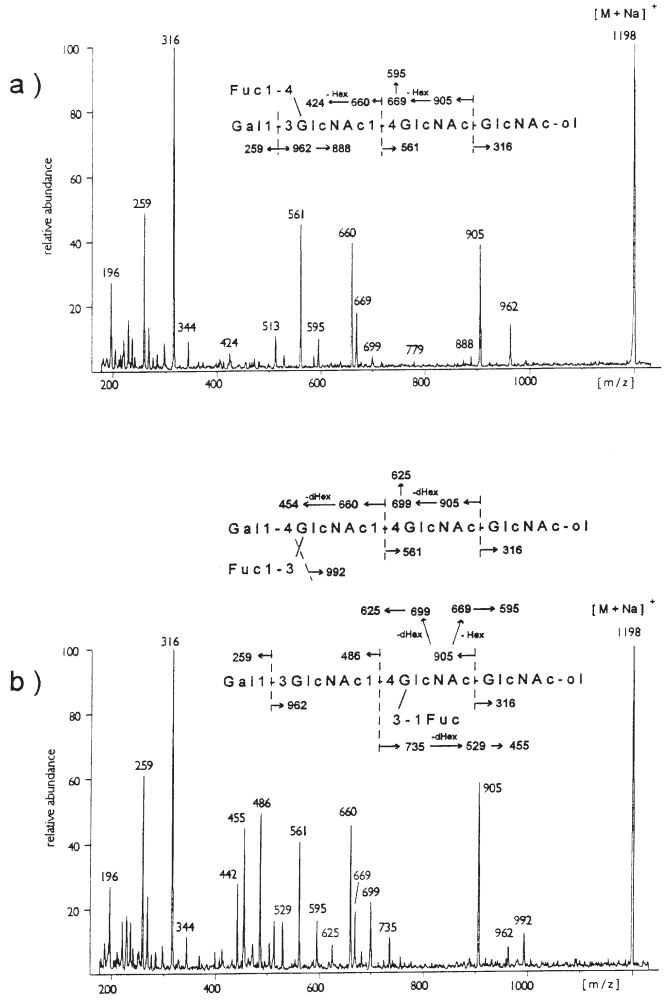

Figure 4. ESI-MS/MS of the sodium adducts of reduced and permethylated monofucosylated incubation products of the 3/4galactosylated B.1+B.2 with FucT III (panel A) and FucT VI (panel B). The daughter ion spectra allow a ready distinction between the oligosaccharide bearing a Le^a motif Galβ1-**3**(Fucα1-**4**)GlcNAcβ1-4GlcNAcβ1-4GlcNAc-ol (A) from the isomeric compound with a Le^x motif Galβ1-**4**(Fuc1-**3**)GlcNAcβ1-4GlcNAcβ1-4GlcNAc-ol (B) as is explained in the affixed fragmentation schemes. The fragmentation pattern of spectrum b, however, can only be completely explained by the presence of an additional isomer Galβ1-**3**GlcNAcβ1-4(Fucα1-**3**)GlcNAcβ1-4GlcNAc-ol (ca. 33%) bearing fucose linked to the middle GlcNAc residue as is demonstrated by the mutually exclusive fragment ion pairs at m/z 561 [HexNAc-HexNAc-ol] and 735 [dHex HexNAc HexNAc-ol] and the complementary pair from the nonreducing end of the molecule at 660 [dHex Hex HexNAc] and at 486 [Hex HexNAc]. The latter isomer must bear the galactose residue linked to O-3 of the subterminal GlcNAc residue, since the respective fragment at m/z 962 generated by elimination of the three-linked galactose is clearly visible in addition to the analogous fragment at m/z 992 generated by the loss of the three-linked fucose from the dominant isomer.

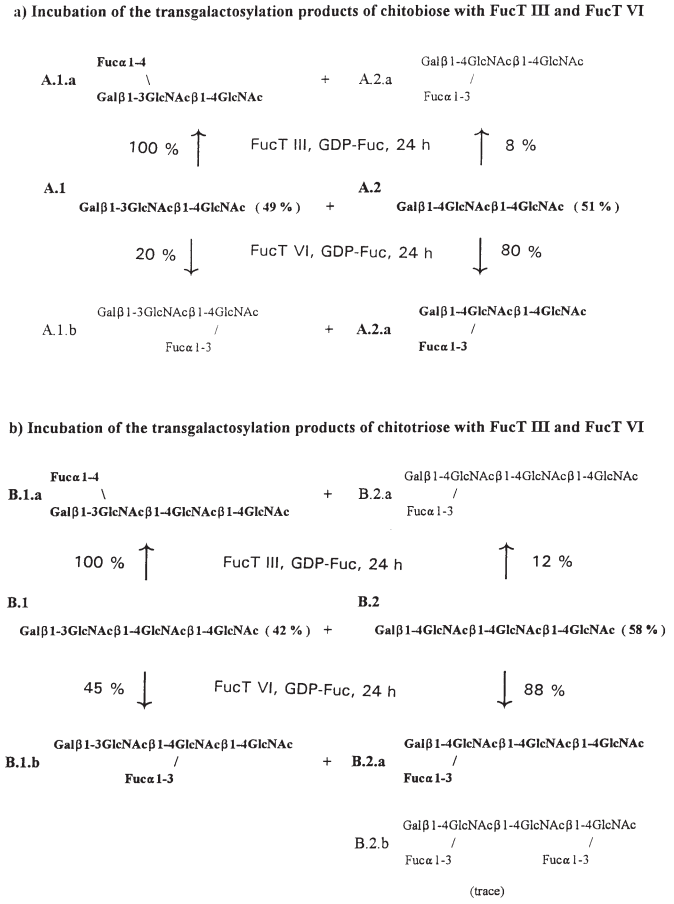

Figure 5. Scheme of the reaction products of approximately equimolar mixtures of type I and type II *N*-acetyllactosamine derivatives [A) Gal3/4(GlcNAc)$_2$, B) Gal3/4(GlcNAc)$_3$] after 24 h incubations with large amounts of recombinant human FucT III or FucT VI in the presence of GDP-fucose. Quantitation of oligosaccharides was performed by peak integration after HPAEC-PAD (compare Figure 1) and confirmed by integration of the anomeric proton signals in the ^{1}H NMR spectra, as well as the relative molecular ion intensities in ESI-mass spectra. Values for the reaction products given correspond to % conversion of each individual isomer for each fucosyltransferase incubation. Major products are printed in bold letters.

fucosylated oligosaccharide peak at m/z 953, however, yielded two series of mutually exclusive fragments, that is the fragment characteristic for unsubstituted proximal GlcNAc at m/z 316 was detected together with the fragment for the respective fucosylated species at m/z 490, confirmed by detection of the complementary fragment ions at m/z 660 [Hex dHex GlcNAc] and 486 [Hex HexNAc] from the nonreducing end of the molecule. This fragmentation pattern can only be explained by the presence of two isomers. The relative intensities of the fragments suggest a ratio of approximately 4:1 for the Le^x type structure **A.2.a** to the isomer fucosylated at the proximal GlcNAc **A.1.b** (compare Figure 4B). Methylation analysis data (Table 1) corroborated this interpretation as the detection of the derivative characteristic of 3,4-disubstituted reducing GlcNAc clearly demonstrated the presence of an oligosaccharide bearing a fucose residue at *O*-3 of the proximal GlcNAc. The linkage of the terminal galactose of this isomer is predominantly to O-3, since the small

amounts of 4-substituted GlcNAc detected by methylation analysis must be attributed to the approximately 10% of unreacted **A.2** detected by HPAEC-PAD (Figure 1c).

For the oligosaccharide mixture **B.1**+**B.2**, as 88% conversion of **B.2** to the expected Le^x oligosaccharide **B.2.a** after 24 h incubation with FucT VI was observed by HPAEC-PAD (Figure 1F; Figure 5B, lower panel). Interestingly, however, 45% of the type I oligosaccharide **B.1** was also converted to a fucosylated product **B.1.b,** giving rise to a well-resolved additional peak in the chromatogram. After reduction and permethylation of the desalted total oligosaccharide mixture, molecular ions at m/z 1024 [Hex $HexNAc_2$ HexNAc-ol+Na] (30%), 1198 [Hex dHex $HexNAc_2$ HexNAc-ol+Na] (70%), and at 1372 [Hex $dHex_2$ $HexNAc_2$ HexNAc-ol+Na] (trace) were detected by ESI-MS. CID of the unreacted species demonstrated again the presence of predominantly type I material **B.1,** and the daughter ion spectrum of the monofucosylated derivative (Figure 4B) indicated the presence of two major isomers. Whereas the fragment ion characteristic of a fucosylated proximal GlcNAc at m/z 490 was not detected, excluding a proximal fucosylation of both isomers, the respective fragment ions incorporating two unfucosylated GlcNAc residues at m/z 561 [HexNAc-HexNAc-ol+Na] and the respective monofucosylated species at m/z 735 [dHex-HexNAc-HexNAc-ol+Na] and the complementary fragments at m/z 660 [(dHex)HexHexNAc+Na] and 486 [HexHexNAc] conclusively demonstrated the presence of a mixture of two isomers bearing a fucose residue at the subterminal GlcNAc leading to a Le^x motif (**B.2.a**) or at the inner GlcNAc residue (**B.1.b**) (see fragmentation scheme at Figure 4B). Since, in addition to the fragment due to the elimination of 1-3-linked fucose from the dominant isomer **B.2.a** at m/z 992, the respective signal generated by the loss of galactose at m/z 962 is clearly visible, this residue must be linked predominantly 1-3 to the subterminal GlcNAc residue in **B.1.b**, as a 1-4-linked sugar residue would not be eliminated. Only trace amounts of the derivative indicating a 1-3-fucosylation at the proximal GlcNAc were detected by methylation analysis (Table 1), confirming the predominant fucosylation of the two inner GlcNAc residues. Interestingly, the difucosylated derivative **B.2.b** present in trace amounts was shown to bear a fucose residue at the proximal and third GlcNAc residue as could be unequivocally deduced from the intense fragment ion series at 490 [dHex HexNAc-ol+Na], 735 [HexNAc (dHex)HexNAc-ol+Na], and 660 [(dHex)HexHexNAc+Na] (data not shown).

In vitro fucosylation of commercial oligosaccharide substrates and complex type biantennary oligosaccharides

The activity of FucT III and FucT VI with LNFP-I, LNT, LNnT, LST-a and SLN was tested as described under Materials and Methods at 0.32 mM concentrations. Complex type *N*-glycans were used at 80 μM concentration. Integration of peak areas after HPAEC-PAD analysis (Table 3) indicated that only LNnT was recognized by FucT VI, whereas the complex-type glycans were efficiently fucosylated. The disialylated diantennary type II oligosaccharide containing two α2-3-linked NeuAc was converted into the Le^x derivatives with peripheral fucose at either one or two antennae. The respective molecular ions were detected by MALDI/TOF MS and their identity was confirmed by methylation analysis (data not shown). The asialo diantennary glycan was found to be modified less efficiently than the sialylated form.

FucT III showed virtually no activity toward complex-type oligosaccharides with type II chains under the same incubation conditions. However, small type I oligosaccharides LNFP-I, LST-a, and LNT were modified [8] yielding Le^a related structures, whereas LNnT was fucosylated at the reducing Glc residue only.

Table 3. Fucosylation of commercial low molecular weight and complex type oligosaccharides by recombinant human FucT III and FucT VI. Incubations contained the standard enzyme concentration determined with the 8-methoxycarbonyloctyl type I or II glycoside acceptor, which yielded 2 nmol of respective product formed within 1 hour of incubation (see Materials and Methods)

	% Fucosylation	
Oligosaccharide acceptors:	*FucT III (after 4 h)*	*FucT VI (after 21 h)*
LNFP	49	0
LNT	20	0
LNnT	13[a]	18
LST-a	19	0
SLN	0	0
diantennary asialo N-glycan	0	14
diantennary disialo N-glycan (NeuAc α2-3)	0	70[b]

[a]LNnT was fucosylated exclusively at the reducing Glc [see reference 8].
[b]Yielded peripherally mono- and difucosylated product in a ratio of approximately 2:3.

Discussion

Transgalactosylation of chitobiose and chitotriose using bovine testis β-galactosidase afforded mixtures of β1-3/4 galactosylated tri- and tetrasaccharides in ratios of 1:1 and 1:1.4, respectively, after purification on Biogel P2. This result is compatible with the substrate specificity of this galactosidase, which is known to act on β1-3 as well as β1-4 linked galactose in glycoconjugates.

In the present study, we have used these two purified oligosaccharide mixtures for studying the applicability of recombinant human FucT III and FucT VI for the *in vitro*

synthesis of Le^a and Le^x carbohydrate structures thereof. Employing the roughly equal mixture of the type I and type II of either the tri- or or tetrasaccharides, together with each of the fucosyltransferases in single incubations, should give information on the substrate preferences of the two enzymes for each isomeric oligosaccharide.

Although differences in the substrate specificity of human FucT III have been reported in the literature, these may be explained by differences in substrate or enzyme concentrations as well as the enzyme source that were used [5–9; see also introduction].

Our previous work has shown that the secreted form of recombinant human FucT III transfers fucose to position O-4 of GlcNAc yielding only Le^a motifs and no Le^x motifs with small oligosaccharides and complex-type N-glycans [8]. Here we have used 20 to 40-fold higher enzyme activities of FucT III in long-term incubations, together with high substrate concentrations (> 3 mM) to detect side specificities which could be advantageously used for the *in vitro* synthesis of fucosylated carbohydrate derivatives.

After 24 incubations of human FucT III with either the Galβ1-3/4GlcNAcβ1-4GlcNAc or Galβ1-3/4GlcNAcβ1-4GlcNAcβ1-4GlcNAc substrate mixture in the presence of 5 mM GDP-fucose, a high conversion of the type **I** isomer into the Le^a derivative was observed as expected. In contrast to our previous findings with small oligosaccharides and asialo-fetuin, some 10% of α1,3-fucosylation of the type **II** Galβ1-4$(GlcNAc)_2$ and Galβ1-4$(GlcNAc)_3$ compounds were detected at the high concentrations used here. No modification of any other than the galactosylated GlcNAc moiety could be identified by tandem mass spectrometry and NMR techniques. Under the incubation conditions used in our previous study employing 0.33 mM concentrations of LNnT, only fucose transfer onto the reducing O-4 substituted Glc was detected, and no Le^x structure was formed [8].

Data from the literature for human FucT VI indicate that the enzyme acts only on type **II** oligosaccharides with or without α2-3-linked NeuAc yielding Le^x or Le^x structures [10, 19,20,23]. In the present study, under identical incubation conditions that were used for FucT III, we observed large amounts of the Le^x product from FucT VI for both the Gal$(GlcNAc)_2$ and Gal$(GlcNAc)_3$ incubations, whereas the type **I** substrate isomer of the galactosylated chitobiose was found to be fucosylated at the proximal GlcNAc only. In the case of the Galβ1-3$(GlcNAc)_3$ substrate, the GlcNAc residue two was fucosylated (about 45% conversion), and no detectable modification of the reducing *N*-acetyl amino sugar occurred. The type **II** isomer was found to be fucosylated in large amounts to yield the corresponding Le^x derivative. In addition, the application of highly sensitive collision-induced decomposition mass spectrometry allowed detection of small amounts of a difucosylated Le^x structure with the second fucose linked α1-3 to the proximal GlcNAc for the Galβ1-4$(GlcNAc)_3$ substrate, yielding a structural motif characteristic for plant and insect *N*-glycans.

Thus, recombinant human FucT VI has a strict requirement for O-4 substituted GlcNAc and does not act on Galβ1-3GlcNAc-R structures. This is in agreement with the results obtained with LNT, LNFP-I, and LST-a where no products were detected for interaction at the GlcNAc or at the reducing glucose residue by FucT VI. This contrasts to the situation with recombinant human FucT III where LST-a, LNT, and LNnT were fucosylated in position O-3 of the reducing glucose residue.

The tandem mass spectrometric techniques applied in this study were shown to represent a sensitive and valuable tool not only for the characterization of purified oligosaccharides, but also for the rapid identification and characterization of the products and unreacted substrates resulting from incubation mixtures. From the fragmentation pattern, the linkage position of fucose to the various GlcNAc residues of the enzymatically synthesized compounds could be directly deduced, and even mixtures of isomers with fucosylation at different GlcNAc residues of the chitobi- or triose core could be directly characterized by the series of mutual exclusive fragment ion pairs. Furthermore, the differentiation between isomers with 3- or 4-substituted GlcNAc, that is between Le^a and Le^x structural motifs is achieved by the analysis of secondary fragment ions by taking advantage of the preferred elimination of the 3-linked compared to the 4-linked substituent [24]. However, the applied MS/MS techniques are less rigorous for structural elucidation than the 1D and 2D NMR techniques used for the characterization of the purified FucT III products.

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