Stable BHK-21 cell lines were constructed expressing the Golgi membrane-bound form and two secretory forms of the human α1,3/4-fucosyltransferase (amino acids 35–361 and 46–361). It was found that 40% of the enzyme activity synthesized by cells transfected with the Golgi form of the fucosyltransferase was constitutively secreted into the medium. The corresponding enzyme detected by Western blot had an apparent molecular mass similar to those of the truncated secretory forms.

The secretory variant (amino acids 46–361) was purified by a single affinity-chromatography step on GDP-Fractogel resin with a 20% final recovery. The purified enzyme had a unique NH2 terminus and contained N-linked endo H sensitive carbohydrate chains at its two glycosylation sites. The fucosyltransferase transferred fucose to the O-4 position of GlcNAc in small oligosaccharides, glycolipids, glycopeptides, and glycoproteins containing the type I Galβ1–3GlcNAc motif. The acceptor oligosaccharide in bovine asialofetuin was identified as the Man-3 branch triantennary isomer with one Galβ1–3GlcNAc. The type II motif Galβ1–4GlcNAc in bi-, tri-, or tetraantennary neutral or α2–3/α2–6 sialylated oligosaccharides with or without N-acetyllactosamine repeats and in native glycoproteins were not modified.

The soluble forms of fucosyltransferase III secreted by stably transfected cells may be used for in vitro synthesis of the Lewisα determinant on carbohydrates and glycoproteins, whereas Lewisα and sialyl-Lewisα structures cannot be synthesized.

The Lewis blood group α1,3/4-fucosyltransferase (FucT-III) has been reported to catalyze the formation of the Lewisα (Leα), Lewisβ (Leβ), sialyl Lewisα (sLeα), and sialyl Lewisβ (sLeβ) determinants (1). The determinant sLea is found on glycoproteins and glycolipids at the surface of leukocytes and tumor cells (2), whereas sLeα is mostly found at the surface of cancer cells of the digestive system (3). It has been found that both determinants are components of ligands for L-, E-, and P-selectins (4, 5). During inflammatory processes, the leukocytes interact with E-selectin induced at the surface of endothelial cells via the sLeα structure (1) and adhesion between tumor cells and the endothelium is mediated by the sLeα and the sLea structures (3). Molecules containing the sLeα and sLea determinants at their surface can be efficiently used for inhibition of cellular interactions and, consequently, are potential tools for the therapeutic treatment in inflammatory diseases and in metastasis. The recombinant FucT-III or soluble variants have been reported to synthesize the two structures in vitro (6–9).

The purification of FucT-III from human milk and from the supernatant of the human epidermidic carcinoma cell line A431 has been reported (6, 8). Soluble chimeric forms of FucT-III fused to a sequence of protein A via the amino terminus have been characterized from transiently transfected COS cells (9, 10). Solubilization of the enzyme due to the deletion of its cytoplasmic domain, transmembrane, and part of the stem region has been reported to change its enzymatic properties. The activity with type II glycolipid acceptors is decreased to non-detectable levels, and the activity with glycoprotein acceptors, particularly asialofetuin, is increased (9).

In the present work, we describe the construction of plasmids encoding soluble forms of the recombinant human FucT-III, where the human IL-2 signal sequence is linked to Ala-47 or Val-36 of the FucT-III and their expression from stably transfected BHK-21 cell lines. The purification of a secreted enzyme form by a single-step affinity chromatography procedure using GDP-Fractogel from supernatants of recombinant stable BHK-21 cells, which do not express any Lewis-type fucosyltransferase activity in their wild-type form, is described for the first time. The purified recombinant enzyme was characterized, and its specificity toward the acceptor substrate motif in the N-glycans of bovine asialofetuin is reported.

**EXPERIMENTAL PROCEDURES**

**Materials**—GDP-fucose, bovine asialofetuin, native fetuin, and bovine thyroglobulin were purchased from Sigma. GDP-[14C]fucose (285 mCi/mmol) was from Amersham (Braunschweig, Germany [FRG]). Recombinant antithrombin III expressed from CHO cells, β-trace protein, and erythropoietin from BHK-21 cells were purified and characterized.
as described previously (11, 12, 13). GDP-Fractogel was from Merck, Darmstadt, FRG. The soluble form of recombinant human IL-2 receptor expressed from CHO cells was a gift from Dr. G. Zettlmeißl (Behringwerke Marburg, Germany). Lacto-N-tetraose (LNT, GallGlcNAcβ3GlcGalβ4Glc), lacto-N-neotetraose (LNNt, GallGlcNAcβ3Glcβ3Glcβ3Galβ4Glc), lacto-N-tetraose II (LNTII, GallGlcNAcβ3Glcβ3Glcβ3Galβ4Glc), lacto-N-trisaccharide a (Lst-a, NeuAca2-3GallGlcNAcβ3Glcβ4Glc), and 3'-sialyl-N-acetyllactosamine (SLN, NeuAca2-3GallGlcβ4GlcNAc) were bought from Oxford Glycosystems, UK. N-Linked complex-type bi-, tri-, and tetraantennary structures with one to three N-acetyllactosamine repeats and one to four α,2,3-linked NeuAca residues were isolated from recombinant glycoproteins expressed from CHO cells and were structurally characterized by NMR and mass spectrometric techniques as described previously (11–15). Purified anti-human Fuc-TIII antibody was a gift from Dr. J. Lowe (University of Michigan Medical School). The monoclonal antibody A3C5 was a gift from Dr. W. Lindenaefer (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany). The GallGlcNAc β-OCH3,COOMe and GallGlcNAcβ-OCH3-H β COOMe acceptors were a gift from Dr. O. Hindsgaul (University of Alberta, Canada). The glycoplipid Gall1,3Glcβ1,3Gall1,4Glc- ceramide was obtained from Dr. K. Bniep (University of Leipzig, Germany), and the glycopeptide ASITTNT(NaGall, 3GlcNaβ1)-Y was a gift from Dr. Kunz (University of Mainz, Germany). Peptide-N'-N(γ-glutamyl-β-glucosaminy] asparagine amidase (PNGase F) (recombinant enzyme from Flavobacterium species) was purchased as an ascites extract and endoglycosidase H was purchased from Boehringer (Mannheim, Germany), Vibrio cholerae sidialase was from Calbiochem (La Jolla, CA), HPLC-grade trifluoroacetic acid and acetonitrile were from Pierce and J.T. Baker (Deventer, The Netherlands), respectively, purumycin- dithydrochloride was bought from Sigma (Deisenhofen, Germany). DMEM was prepared using Dulbecco's modified Eagle's medium (purchased prior to use with 10 mM Hepes, 45 mM NaHCO3, 2 mM glutamine, 0.061 g liter\(^{-1}\) ampicilline and 0.1 g liter\(^{-1}\) streptomycin sulfate and which was adjusted to pH 7.1 for use in cell culture.

Construction of a \(\alpha,1,3,4\)-Fucosyltransferase Variants—Mutants of the human \(\alpha,1,3,4\)-fucosyltransferase were generated by PCR-based site-directed mutagenesis of a Fuc-T III cDNA (a gift from Dr. S. Gonski, Hoechst AG, Frankfurt, FRG) encoding the full length of the enzyme. PCR was performed using the Expand High Fidelity DNA-polymerase mixture (Boehringer) and the supplied buffer according to the manufacturer's protocol at standard concentrations of 0.3 \(\mu\)M for each primer and 0.2 \(\mu\)M each deoxynucleotide. PCR conditions, if not otherwise stated, were a 2-min denaturation step at 94 °C, followed by 30 cycles of: amplification at 94 °C, 20 s of annealing at 50 °C, 2 min of elongation at 72 °C, and final elongation for 8 min at 72 °C. DNA fragments were cloned into the eukaryotic expression vector pCR3 using a TA cloning kit (Invitrogen, Leek, The Netherlands). Positive clones were identified by using standard techniques and were verified by using the CircumVenit Thermal Cycle Dideoxy Sequencing kit (New England Biolabs, Schwalbach, Germany).

The mutant pCRS1FT3 encodes a full-length human Fuc-T III that is COOH-terminally elongated with a tripeptide-spacer GAG followed by the epitope FDKNYANSKG derived from human cytomegalovirus \(\alpha\)-2-deoxy-

Fucosyltransferase Assays—The fucosyltransferase activity with oligosaccharides was determined as described in 12.

Preparation of Cell Extracts—Cell extracts were freshly prepared and thawed with frozen 

Enzyme Purification—The culture supernatants were a 20-ml Mops-KOH buffer, pH 7.5, containing 2% Triton X-100 \(\times 10^3\) cells) cells were disrupted using a Potter-Ehrlheim homogenizer at pH 6. If not otherwise stated, extracts were used within the same day of homogenization for fucosyltransferase activity determinations.

SDS-PAGE and Western Blot Analysis—SDS-PAGE was performed according to Laemmli (17) using 12.5% and 3% acrylamide in the resolution and stacking gels, respectively. For Western blot analysis, proteins were transferred to nitrocellulose (Millipore) in a semidry instrument (Bio-Rad). The membrane was blocked with Tris-buffered saline containing 10% horse serum and 3% bovine serum albumin for 1 h and incubated overnight with biotinylated anti-cytomegalovirus tag monoclonal antibody (A3C5) or anti-FucT-III antiserum in blocking buffer at 1:1000 dilutions, respectively. The second antibody, streptavidin or rabbit-antibunmodinoligobond to horseradish peroxidase, respectively, was used at a 1:1000 dilution. The blots were developed with Tris-buffered saline containing 0.5 mg ml\(^{-1}\) 4-chloro-1-naphthol solubilized in methanol and 0.2% perhydrol. Endoglycosidase H II as well as mild acid treatment were performed as described (12).

Fucosyltransferase Assays—The fucosyltransferase activity with oligosaccharides, glycolipids, glycopeptide, and 8-methoxycarbonylglucicyl aspartate primers type I and II was tested at 37 °C in 60 μl of the following reaction mixture using 20–60 microunits of enzyme; 50 mM Mops/NaOH buffer, pH 7.5, 20 mM Mclα, 0.1 mM NaCl, 4 mM ATP, 0.1 mM GDP, [\(^{14}\)C]Fuc (containing 60,000 cpm of GDP-[\(^{14}\)C]fucose/nmol). Glycolipid and glycopeptide acceptors were added to 1 and 8-methoxy carbonyl glycodeoxyacetate acceptors at 0.3 mM concentrations. SLN, LNT, LNT, and LNT-a were used at a 0.33 mM concentration in the presence of 4.2 mM GDP-Fuc. Reducing N-glycans of the bi-, tri- and tetra-antennary complex and bi-, tri- and tetra-antennary complex and bi-, tri- and tetra-antennary complex N-glycan were detected and used as acceptors in the presence of 0.2 mM GDP-Fuc for 4 and 24 h. Aliquots of reaction mixtures with oligo saccharides and N-glycans were analyzed by HPAE-PAD after desialylation with V. cholerae sidialase and by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI/TOF-MS) after desialylation. Some of the samples for MALDI/TOF-MS analysis were reduced and

\footnote{W. Lindenaefer, personal communication.}
permutated. The reaction mixtures with glycolipid and 8-methoxy-
arylobutylcylectoside as acceptors were diluted with water to 1 ml and applied to Sep-Pak C18 cartridges, which were washed with 5 ml of water. The products were then eluted with 1 ml of methanol. The incorporation of $^{14}$C-fucose was determined by liquid scintillation counting. The glycopeptide reaction mixtures were precipitated for 10 min with 1 ml of 1% phosphotungstic acid in 0.5 M HCl and transferred under vacuum to glass microfiber filters (Whatman GF/C). Filters were washed with 1% tungsphoric acid in 0.5 M HCl and methanol, dried, and counted for radioactivity in a Beckman LS 6000 LC scintillation counter. There was no incorporation of Fuc in reaction mixtures with enzyme without or without acceptor. For a detailed characterization of the fucosylated N-linked oligosaccharides from asialofetuin, 10 mg of the protein were incubated with S2FT3T2 in the buffer described above, containing 750 nmol of GDP-$^{14}$CFuc for 24 h at 37 °C. The resulting protein was dialyzed against 0.5% acetic acid, lyophilized, and the corresponding oligosaccharides were released by automated hy-
trations of the Analyte mixtures were approximately 10 pmol

**RESULTS**

**Construction and Isolation of Stable BHK-21 Cells Expressing Human a1-3/4 Fucosyltransferase Activity—BHk-21B**

Cells were transfected with plasmids encoding the membrane-
bound form of FucT-III with a tag at its carboxyl terminus (FT3T2), and two soluble forms of FucT-III containing the human IL-2 leader peptide sequence, at amino acid 36 (SFT3T3) or amino acid 47 (S2FT3T2, with a tag at its COOH terminus) (Fig. 1). After transfection, cells were selected in medium containing 0.8 μg ml$^{-1}$ puromycin hydrochloride. Resistant cell clones were isolated, and, after reaching confluence, supernatants and cellular extracts were assayed for fucosyltransferase activity with Galβ3–6GlcNAc-O(CH$_2$)$_2$COOMe as an acceptor substrate (Table I). The highest activity of secreted FucT-III was measured in the supernatant of stable BHK-21 cells transfected with pCRS2FT3T2. Secretion of enzyme activity increased for up to 40 h in confluent cultures supplemented with fresh medium. This clone (S2FT3T2) was used for the production and purification of the recombinant enzyme (see below).

For the cell clones expressing secretory forms of FucT-III, some fucosyltransferase activity was detected in the cellular extracts (10–20% of the secreted form, see Table I) presumably repre-
senting the enzyme fraction transported along the secretory pathway of cells. For cells transfected with the membrane-
bound form of FucT-III, most of the activity was found to be associated with cellular extracts; however, considerable amounts (about 40% of the total activity measured with Galβ3–6GlcNAc-O(CH$_2$)$_2$COOMe as a substrate) were detected in the culture supernatant of confluent monolayers (viability higher than 95% based on trypan blue exclusion) that were cultivated for a 24-h period in fresh medium. The Western blotting analysis of concentrated supernatants from the three cell lines using the anti-FucT-III antibody showed bands of similar molecular masses around 40 kDa. This indicates a significant shedding of the membrane-bound form (FT3T2), presumably after proteolytic cleavage within the stem region.

**Purification and Characterization of the Soluble Form of the Recombinant Human Fuc-T III**—For the purification of S2FT3T2, confluent recombinant monolayers were grown for 4 weeks in DMEM containing 2% FCS, the medium being har-
vested every 2 or 3 days and being frozen at −20 °C until further use. The enzyme was purified from 1600 ml of culture supernatant by affinity chromatography on a GDP-Fractogel column using the one-step procedure as described under “Ex-
perimental Procedures.” The eluate containing the S2FT3T2 was concentrated by ultrafiltration and was stored in the presence of 30% (v/v) glycerol. The activity of the pooled superna-
tants after thawing decreased by approximately 2.5-fold. The purified S2FT3T2 preparation (4.5 ml) had a protein concentration of 30 μg ml$^{-1}$ and an activity of 33 units/liter with the type 1 8-methoxyarylobutylcylectoside as a substrate). Thus, the final recovery of recombinant S2FT3T2 was approximately 20%.

Upon SDS-PAGE analysis followed by Coomassie staining, the enzyme preparation was found to consist of a closely spaced doublet of about 40–42 kDa molecular mass, which was also detected with the anti-Fuc-TIII antibody as well as with the
S1FT3:

|         | IL-2 | Fuc-TIII |
|---------|------|----------|
| Control | 17p  | 42       |
| S1FT3   | 17p  | 42       |
| FT3T2   | 17p  | 42       |
| Control | ND   | ND       |

S2FT3T2:

|         | IL-2 | Fuc-TIII |
|---------|------|----------|
| Control | 13   | 19       |
| S2FT3T2 | 13   | 19       |
| FT3T2   | 13   | 19       |
| Control | ND   | ND       |

**TABLE I**

| Fuc-TIII construct | [14C]Fucose |
|--------------------|------------|
|                     | cpm/6 h    |
| Culture medium      | Cell extract |
|---------------------|-------------|
| S2FT3T2             | 38,700      |
| S1FT3               | 1,000       |
| FT3T2               | 13,600      |
| Control             | ND          |

**Fig. 1.** Schematic presentation of the Fuc-TIII constructs used for stable transfection of BHK-21 cells.

Human fucosyltransferase activity in supernatants and cellular extracts from stable BHK-21 cells transfected with pCRSFT3T2, pCRS1FT3, or pCRFT3T2

Thirty nmol of Galβ1,3GlcNAc-O-(CH2)8COOMe as acceptor substrate and 30 nmol of GDP-Fuc (100,000 cpm) were used for up to 24 h. Activity was determined in comparable amounts of concentrated supernatants and 30 nmol of GDP-Fuc (100,000 cpm) were used for up to 24 h. The apparent kinetic parameters determined with the type I acceptor using saturating concentrations of the GDP-Fuc were \( V = 0.83 \pm 0.07 \text{ pmol min}^{-1} \text{ml}^{-1} \), and \( K_m = 0.54 \pm 0.08 \text{ m}\), assuming a Michaelian behavior for the enzyme.

The specificity of the S2FT3T2 toward the small oligosaccharide acceptors LNFP, LNT, LNNT, LST-a, and SLN was analyzed after incubations of 2, 4, and 21 h. The formation of the fucosylated products was monitored by HPAE-PAD (Fig. 3), and the molecular masses of the products were determined by MALDI/TOF-MS (Table II). It was found that the reaction was linear for at least 4 h, so the activities shown in Table II were calculated based on 2-h incubation values. The S2FT3T2 activity with the type I acceptor (LNT) is 1.5-fold higher than with the type II acceptor (LNNT). Substitution of the terminal monosaccharide residue of the LNT with α,2,3-linked sialic acid causes a 1.3-fold increase in activity of the S2FT3T2. Substitution of the LNT with α,2-linked Fuc causes a 3.3-fold increase in activity of the S2FT3T2 (Table II). To identify the linkage position of fucose residues, after reduction and permethylation, the oligosaccharides were analyzed by collision-induced decomposition mass spectrometry and by methylation analysis (Fig. 3 and Table III). For LNT, it was found that S2FT3T2 transferred Fuc to the O-4 position of GlcNAc (70%), to the O-3 position of Glc (20%), or to both positions (10%). In LNNT, all the Fuc was found to be attached to the O-3 position of Glc. With LNFP as an acceptor, Fuc was transferred exclusively to the O-4 position of GlcNAc with no fucosylation being detected on the Glc residue. 80% of the product obtained with LST-a contained fucose at the O-4 position of GlcNAc; 20% was modified at the O-3 position of Glc; additionally, based on the molecular ions detected in ESI spectra, small amounts of bifucosylated and trace amounts of trifucosylated structures were observed in the product with this substrate (compare Table II).

No fucosylation was detected in the trisaccharide SLN when incubated under identical conditions.

S2FT3T2 activity toward glycolipids was tested with Galβ3GlcNAcβ3Galβ4Glcβ1–1ceramide as a substrate. It was found that 63 pmol/min/ml of Fuc transferred to 50 nmol of the acceptor.

The chemically synthesized glycopeptide ASTTTN-(βGal1,3βGlcNAc1)YT (mass of 1243) was almost totally fucosylated by S2FT3T2 during an overnight incubation, yielding a product with \( m/z = 1389 [M + Na]^+ \) as detected by ESI-MS.

Complex-type bi-, tri-, and tetraantennary structures with zero to four N-acetyllactosamine repeats and with or without α,2,3/6-linked NeuAc residues were incubated with the enzyme for 4 and 24 h. Analyses by HPAE-PAD and MALDI/TOF-MS revealed that no fucosylation had occurred irrespective of the antennarity, number of lactosamine repeats or sialylation degree after a 24-h incubation period.
The glycoproteins, bovine asialofetuin, native fetuin and bovine thyroglobulin, human \( \beta \)-trace protein from hemofiltrate (20), recombinant human \( \beta \)-trace protein, recombinant human antithrombin III, and recombinant human IL-4 receptor from CHO cells, were tested as acceptors for S2FT3T2 (Table IV) by determination of \([14C] \)fucose incorporation. Only very low incorporation of fucose was achieved. The enzyme showed the highest activity with asialofetuin as a substrate. The activity with recombinant \( \beta \)-trace protein from BHK-21 cells was higher than with its natural counterpart isolated from hemofiltrate (15). No fucosylation of bovine thyroglobulin or recombinant antithrombin III from CHO cells was detected. Fucosylated glycoproteins were subjected to SDS-PAGE, and, following subsequent autoradiography, radiolabeled bands were detected at migration positions corresponding to the molecular masses of the untreated glycoproteins.

**N-Glycan Structures of in Vitro Fucosylated Bovine Asialofetuin**—For the determination of the linkage position of fucose in asialofetuin oligosaccharides, 10 mg of the glycoprotein were incubated with S2FT3T2 in the presence of 750 nmol of GDP-[\( ^{14} \)C]Fuc (2 \( \times 10^6 \) cpm) for 18 h. The oligosaccharides from the unmodified and modified glycoprotein were released by automated hydrazinolysis and subjected to HPAEC-PAD (Fig. 5). Three major oligosaccharide peaks were obtained for the glycan mixture from unmodified asialofetuin: biantennary, triantennary 2,4-branched, and triantennary 2,4-branched with one Gal\( \beta 1–3 \)GlcNAc antenna in a ratio of 10:55:35. The glycan mixture from \textit{in vitro} fucosylated asialofetuin yielded a new peak eluting at 15.5 min (C1) with a concomitant decrease of peak C (C1, A, B, C in ratio of 27:11:53:9). The glycan mixture from S2FT3T2-treated asialofetuin was subjected to separation on NH\(_2\)-bonded phase. Two major peaks were obtained, which were not completely separated (data not shown). Two molecular ions corresponding to reduced and permethylated triantennary \( N \)-glycans (m/z 52537) and a fucosylated triantennary structure (m/z 52710), respectively, were detected after MALDI/TOF-MS. The native material obtained after NH\(_2\)-bonded phase was subjected to preparative HPAEC-PAD yielding peaks C1 and B (Fig. 5, panel 3). Methylation analysis revealed the presence of only 4-substituted GlcNAc in peak B and a mixture of 4-substituted and 3,4-disubstituted GlcNAc as well as terminal fucose in peak C1. Peak C1 eluted at 15.5 min in HPAEC-PAD and was completely converted to a structure eluting at the position of peak C upon mild acid treatment (Fig. 5, panel 4), whereas peak B was not affected. Thus, the results indicate that the \( N \)-linked oligosaccharide of asialofetuin with one type I antenna is modified by S2FT3T2 with fucose linked to position 4 of GlcNAc in the type I motif. No indication of the presence of fucosylated \( O \)-linked glycans was obtained in our experiments.

**DISCUSSION**

The construction of a soluble form of FucT-III through the replacement of amino acids 1–35 and 1–46 by the signal sequence of human IL-2 (constructs S1FT3 and S2FT3T2, respectively) produced catalytically active secreted forms of the enzyme when expressed from stably transfected BHK-21 cell lines.
The purification of human α1,3/4-fucosyltransferase from milk and from the culture supernatant of the A431 carcinoma cell line has been reported by Johnson et al. (6, 8). Their methodology involved four purification steps with a final recovery of approximately 25%. In the present work the purification of a soluble recombinant form of FucT-III was achieved by a single affinity chromatography step using GDP-Fractogel. Comparable final yields were obtained with this procedure.

### Table II

| Oligosaccharide | nmol min⁻¹ ml⁻¹ | Major molecular ion [M + Na]⁺ of products\(^b\) |
|-----------------|-----------------|---------------------------------------------|
| A. Acceptors    |                 |                                             |
| Galβ3GlcnAcβ3Galβ4Glc | 9 (100) | 876, 1023                                    |
| Fuco2Galβ3GlcnAcβ3Galβ4Glc | 33 (367) | 1023                                        |
| NeuAcα2,3Galβ3GlcnAcβ3Galβ4Glc | 12 (133) | 1185                                        |
| Galβ4GlcnAcβ3Galβ4Glc | 6 (67) | 876                                         |
| NeuAcα2,3Galβ4GlcnAc | ND  | ND                                           |
| B. Substrate\(^cd\) |  | 1116 (deoxyHex-Hex-NacHex-01) |
| Galβ3GlcnAcβ3Galβ4Glc (942) | | 1290 (deoxyHex-Hex-NacHex-01) |
| Fuco2Galβ3GlcnAcβ3Galβ4Glc (1116) | | 1291 (deoxyHex-Hex-NacHex-01) |
| NeuAcα2,3Galβ3GlcnAcβ3Galβ4Glc (1303) | | 1477 (NeuAc deoxyHex-Hex-NacHex-01) |
| Galβ4GlcnAcβ3Galβ4Glc (942) | | 1653 (NeuAc deoxyHex-Hex-NacHex-01) |
| NeuAcα2-3Galβ2GlcnAc | ND  | ND                                           |

- \(^a\)Percentage in relation to the LNT substrate is indicated in parentheses.
- \(^b\)Molecular masses of the native products were determined by MALDI/TOF-MS.
- \(^c\)ND, not determined.
- \(^d\)Numbers in parentheses indicate the molecular ion [M + Na]⁺ of the unfucosylated saccharides.
- \(^e\)Signal intensities were <3% of that of the major molecular ion signal.

**FIG. 4.** ESI-MS/MS analysis after reduction and permethylation of LNT products obtained after incubation with S2FT3T2. **a.** LNT (m/z = 942, M + Na)⁺ yielded a monofucosylated molecular ion signal (m/z = 1117) as well as small amounts of bifucosylated structures (m/z = 1291); **b.** fragmentation scheme of the monofucosylated parent molecular ion peak m/z = 1117 (M + Na)⁺ obtained by collision induced decomposition. The two series of mutually exclusive fragment ions depicted by the inset demonstrate the presence of the two isomeric structures with deoxyHex attached either to Hex-Nac or to the proximal Hex-ol.
hydrolysis of NeuAc during reduction and permethylation.

In the A431 carcinoma cells.

a previously for an

occupancy of

glycans that are not decorated with significant amounts of

S2FT3T2 are occupied with oligomannosidic or hybrid-type

acid treatment indicated that the two glycosylation sites of

part of the recombinant enzyme. The results obtained after the

molecular mass is not due to proteolytic degradation at any

quencing of the product, the observed difference in apparent

bands with a unique amino-terminal sequence were detected.

For the purified enzyme described here, two closely spaced

bonds with a unique amino-terminal sequence were detected.

Apparent molecular masses of about 42 kDa were calculated

for asialofetuin oligosaccharides are based on the 2,4-di-

b The values for small oligosaccharide are based on the 2,4,6-tri-

a The values for asialofetuin oligosaccharides are based on the 2,4-di-O-methyl-derivative = 1.0.

TABLE IV

In vitro activity of S2FT3T2 with native glycoprotein acceptors

ND, not detected; see “Experimental Procedures” for details.

| Glycoprotein          | Glycosylation sites | N-Linked oligosaccharide structures | [14C]Fucose transferred |
|-----------------------|--------------------|------------------------------------|------------------------|
|                      | cpm/1 h            |                                    |                        |
| Asialofetuin          | 3                  | Di-, triantennary                   | 5900                   |
| IL-4 receptor (CHO)   | 7                  | Di-, tri-, tetraantennary (7:1:2); α2,3NeuAc | 1900                  |
| β-Trace protein human hemofiltrate | 2 | Diantennary; α2,3-, α2,6-NeuAc | 1900                  |
| β-Trace protein rec. from BHK-21 | 2 | Diantennary; NeuAc | 1240                  |
| Rec. antithrombin III | 4                  | Diantennary; α2,3-NeuAc             | ND                     |

which is more convenient and can also be used for the purification of other recombinant human fucosyltransferases.3 It is also advantageous over other methods, where part of the protein A polypeptide sequence is linked to the amino terminus of the FucT-III, resulting in chimeric enzyme forms that can be purified by adsorption on and elution from IgG-Sepharose. However, in the procedure described in the present work, no fusion with unrelated bulky protein moieties that might alter enzyme specificity toward different substrates (9) is required.

For the purified enzyme described here, two closely spaced bands with a unique amino-terminal sequence were detected. Apparent molecular masses of about 42 kDa were calculated from their mobility in SDS-PAGE and Coomassie staining as well as in Western blotting analysis using an antibody that recognizes the tag sequence fused to the COOH terminus of the enzyme. Since the expected NH2 terminus of the S2FT3T2 polypeptide was unequivocally detected upon gas-phase sequencing of the product, the observed difference in apparent molecular mass is not due to proteolytic degradation at any part of the recombinant enzyme. The results obtained after the incubation with Endo H or PNGase F and neuraminidase/mild acid treatment indicated that the two glycosylation sites of S2FT3T2 are occupied with oligomannosidic or hybrid-type glycans that are not decorated with significant amounts of NeuAc. Based on binding studies with concanavalin A (8), occupancy of N-glycosylation site(s) has been suggested previously for an α1–3/4-fucosyltransferase purified from the culture medium of the A431 carcinoma cells.

The BHK-21 cells stably transfected with the membrane-bound wild-type form of the human FucT-III unexpectedly secreted considerable amounts of a soluble form of the enzyme into the culture supernatant (about 40% of the total activity that was measured after a 24-h production period of confluent growing and more than 95% viable BHK-21 cells). The detection of enzyme activity in supernatants of transiently transfected COS cells has been previously reported by Kukowska-Latallo (10) using expression plasmids encoding the human wild-type FucT-III. The detection of significant enzyme activity in the supernatants of stably transfected BHK-21 (>95% viable) cell clones in the present study suggests a common mechanism of endoproteolytic cleavage of human fucosyltransferase III within the stem region inside Golgi/endoplasmic reticulum compartments, since the apparent molecular weight detected for the secreted form of FT3T2 was very similar to that observed for the truncated genetically engineered secretory variants S2FT3T2 and S1FT3. This would also explain the high amounts of this enzyme that are detected in human milk and supernatants of A431 cells. Recently, Kimura et al. (21) have reported the preparation of a monoclonal antibody against human FucT-III that recognized enzyme forms in Western blotting with approximate molecular masses of 42–45 kDa in a variety of human tumor cell lines including A431 cells. Unfortunately, no data were presented on immunodetection of the FucT-III forms in the culture supernatants of these cells.

The truncated enzyme form S2FT3T2 showed virtually no activity with the type II 8-methoxycarbonyloctyl glycoside acceptor. Similar results were obtained by de Vries et al. (9) when using cell extracts of transiently transfected COS cells with constructs encoding the membrane-bound form of the FucT-III, whereas Johnson et al. (7) measured 10% of enzyme activity.

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8-methoxycarbonyloctyl glycoside acceptor (0.54 mM) is similar to that found for the enzyme purified from human milk (0.5 nmol of radioactive pool (same as reference) per mg of protein A moiety affects the specificity of the enzyme to reduce Glc occurred, and bifucosylated products that contained fucose at the O-4 and O-3 positions of GlcNAc and Glc were detected. These structures were not detected in previously reported work. SLN was not fucosylated by S2FT3T2, in contrast to what was described for Fuc-T-III from milk or secreted from A431 cells.

All glycoproteins tested, except for asialofetuin, were found to be very poor substrates for S2FT3T2 based on [14C]fucose incorporation studies. The asialofetuin used in the present work contained triantennary glycans with terminal Galβ1–3 and Galβ1–4 linked to GlcNAc as described by Rice et al. (23), but no branched O-linked oligosaccharide described by Edge and Spiro (24) was detected. This probably is due to the different commercial origin of the protein preparation used here. The fucosylated N-linked oligosaccharide of asialofetuin was identified as a 2,4-branched triantennary structure containing one Galβ1–3GlcNAc antenna. This type of oligosaccharide is present, if any, in only very small amounts in the other glycoproteins which were used in this work as substrates for S2FT3T2. The observed substrate specificity of the enzyme enables us to suggest that human Fuc-T-III provides a useful tool for detection of type I structures in N-glycosylated glycoproteins.

Based on antibody binding studies, COS-1 cells start to express Leα, α-Leβ, Leβ and sLeβα structures at their surface after transfection with the Fuc-T-III gene (1, 10); some of the fucosylated molecules are glycoproteins (e.g. PSGL-1) (25). However, the secreted Fuc-T-III produced in BHK-21 cells only had the capability of in vitro modifying type I structures, resulting in Leα and sLeα type motifs in small oligosaccharides, glycolipid, glycopeptide, and glycoproteins. In contrast to previously reported work (10), in our study no structure could be detected, indicating that the enzyme recognizes the GlcNAc in type II N-glycans as a substrate; therefore, a biosynthetic involvement of type II N-glycans in the formation of Lewisα or sialyl-Lewisα motifs on glycoconjugates seems questionable. This difference in specificity might be due to the truncation at the amino terminus of the enzyme, or it may result from differences in the intracellular environment/compartimentalization and the in vitro assay conditions applied. However, it should be emphasized that solubilization of membrane-bound glycosyltransferases after disruption of cells in the presence of detergents will frequently result in a mixture of proteolytically cleaved and intact forms, which makes it difficult to unequivocally assess the substrate specificity of the native Golgi enzymes by in vitro assays. Finally, this problem could be solved by coexpression experiments using glycosyltransferase genes and, e.g., secretory model glycoproteins, which must be modified properly by the recipient cell line and must be structurally characterized thoroughly with respect to their carbohydrates. In preliminary studies of our laboratory using coexpression of human erythropoietin and Fuc-T-III in BHK cells, no peripheral fucosylation of the secreted erythropoietin could be detected. This supports our view that the enzyme acts as a α1–4-fucosyltransferase also in vivo.

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