In Vivo Specificity of Human α1,3/4-Fucosyltransferases III-VII in the Biosynthesis of LewisX and Sialyl LewisX Motifs on Complex-type N-Glycans

Coexpression Studies from BHK-21 Cells Together with Human β-Trace Protein

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Each of the five human α1,3/4-fucosyltransferases (FT3 to FT7) has been stably expressed in BHK-21 cells together with human β-trace protein (β-TP) as a secretory reporter glycoprotein. In order to study their in vivo properties for the transfer of peripheral Fuc onto N-linked complex-type glycans, detailed structural analysis was performed on the purified glycoprotein. All fucosyltransferases were found to peripherally fucosylate 19–52% of the diantennary N-glycans, and all enzymes were capable of synthesizing the sialyl LewisX (sLeX) motif. However, each enzyme produced its own characteristic ratio of sLex/Lex antennae as follows: FT7 (1:7), FT6 (14:1), FT5 (3:1), FT6 (1:1:1), and FT4 (1:7). Fucose transfer onto β-TP N-glycans was low in FT3 cells (11% of total antennae), whereas the values for FT7, FT5, FT4, and FT6 cells were 21, 25, 35, and 47%, respectively. FT3, FT4, FT5, and FT7 transfer preponderantly one Fuc per diantennary N-glycan. FT4 preferentially synthesizes di-LeX on asialo diantennary N-glycans and mono-LeX with monosialo chains. In contrast, FT6 forms mostly α1,3-difucosylated chains with no, one, or two NeuAc residues. FT3, FT4, and FT6 were proteolytically cleaved and released into the culture medium in significant amounts, whereas FT7 and FT5 were found to be largely resistant toward proteolysis. Studies on engineered soluble variants of FT6 indicate that these forms do not significantly contribute to the in vivo fucose transfer activity of the enzyme when expressed at activity levels comparable to those obtained for the wild-type Golgi form of FT6 in the recombinant host cells.

The involvement of fucosyltransferases in the biosynthesis of Lewis-type and sialylated Lewis-type carbohydrate structures as ligands for selectins present on endothelial cells, platelets and lymphocytes has been reviewed in several excellent recent reviews (1–4). The interaction of selectins with fucosylated glycoprotein or glycolipid ligands is ascribed a central role in biological phenomena like tumorigenesis, tissue differentiation, and leukocyte adhesion during inflammatory processes; however, the specific role of each of the enzymes within the assembly of these structures is not fully understood.

The in vivo enzyme specificity of the five human α1,3/4-FTs1 (FT3–FT7) that have been cloned so far (5–11) is difficult to assess. Although in vitro activity assays using small oligosaccharide acceptor substrate might be valuable for monitoring the purification of glycosyltransferases from natural or recombinant sources, the substrate specificity determined in vitro might not allow for final conclusions on their in vivo functional role. The structural analysis of a large number of glycoproteins from human tissues indicates a biosynthetic pathway where a sequential and ordered action of glycosyltransferases might be operating (12–14). However, the mechanisms by which this is accomplished in vivo, e.g., how the different subcompartamental localization and temporal action of glycosyltransferases that could compete for the same acceptor substrate in the Golgi is regulated, has not been studied in detail.

Results obtained from in vitro assays of enzymes isolated from tissues or body fluids are often difficult to interpret due to the fact that several enzymes with different or overlapping acceptor substrate specificities might be present. Furthermore, heterogeneity in the polypeptide primary structure of the glycosyltransferase preparations due to different proteolytic cleavage that might occur during tissue/cell disruption procedures must be considered as well when data from the literature are compared. This must also be considered when activity measurements are carried out on recombinantly expressed enzymes from insect or mammalian host cells, which in some cases have been expressed as fusion proteins to facilitate their purification (10, 15–18).

According to the data published so far, FT7 has been re-

1 The abbreviations used are: FT, fucosyltransferase; β-TP, β-trace protein; CHO, Chinese hamster ovary; CTS, cytoplasmic transmembrane, and stem; dHex, deoxyhexose; DHFR, dihydrofolate reductase; DMEM, Dulbecco’s modified Eagle’s medium; Endo H, endo-β-N-acetyl-

1,3/4-galactosaminidase H; ESI, electrospray ionization; ESI-MS/MS, nanospray tandem mass spectrometry; FBS, fetal bovine serum; Galβ3→4GlcNAc-Oct, Galβ3→4GlcNAc-O(CH2)8COOCH3; Galβ4→4-GlcNAc-Oct, Galβ4→4GlcNAc-O(CH2)8COOCH3; Hex, hexose, HexNAc, N-acetyhexosamine; HexNAc-ol, N-acetyhexosaminol; HPAE-PAD, high-pH anion-exchange chromatography with pulsed amperometric detection; HPLC, high-performance liquid chromatography; IL, interleukin; LeX, LewisX (Galβ3→4GlcNAc-R); MALDI/TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; Mes, 2-morpholinoethanesulfonic acid; Mops, 3-morpholinopropanesulfonic acid; NeuAc, N-acetylneuraminic acid; PAG, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PNGase F, peptide-N-ace-

tylglucosaminidase F; sLeX, sialyl LewisX (NeuAcα2→3Galβ3→4[Fucα1→4]GlcNAc-R); sLeX, sialyl LewisX (NeuAcα2→3Galβ3→4[Fucα1→4]GlcNAc-R); ST, sialyltransferase; rhu, recombinant human.

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ported to fucosylate only α,2,3-sialylated, small N-acetyllactosamine-type structures and is inactive with neutral acceptors (10, 11, 18). FT4 acts almost exclusively on uncharged Galβ1→4GlcNAc-R (type II) structures (15, 19), whereas FT5 and FT6 have been reported to act on both α,2,3- and α,2,6-sialylated type II acceptors (15, 16, 20, 21). FT3 has been reported to transfer fucose in α,1,4-linkage onto GlcNAc-R in type I chains (15, 21, 22). Activity with type I acceptors has also been reported for human FT5 (15), while FT4, FT6 and FT7 are not active with Galβ1→3GlcNAc-R substrates (10, 15, 21).

In view of their important biological role in the biosynthesis of selectin ligands by α,1,3/4-FTs, there is a need to define and compare their in vivo specificities toward a suitable acceptor or reporter glycoconjugate that is constitutively expressed and supplied in stable concentrations within the biosynthetic glycosylation pathway. Inflammation-induced expression of SLLEX and Leα on N-acetyllactosamine-type N-glycans of several acute phase proteins in human serum has been reported (23, 24). Walz et al. (25) have shown that in vitro fucosylated human α1-acid glycoprotein leads to interaction with E-selectin expressed on inflamed endothelial cells. These reports have discussed a possible biological role of peripherally fucosylated (acute phase) serum glycoproteins in context with a competitive inhibition of the primary interaction of leukocytes with the selectins (23).

To our knowledge, no reports have been published comparing the in vivo specificity of human α,1,3/4-FTs toward N-glycosylated protein substrates. A recent report by Kimura et al. (26) describes the activity of recombinantly introduced human α,1,3/4-FTs on glycolipids of HeLa and Namalwa cells. In the present paper, we have used stable coexpression of human β-TP together with the full-length forms of the five known human α,1,3/4-FTs from BHK-21 cells in order to assess the in vivo substrate specificity of the different transferases by carbohydrate structural characterization of the secreted β-TP using complementary chromatographic and mass spectrometric techniques. Human β-TP is a 168-amino-acid glycoprotein with two N-glycosylation sites, which has been shown previously to contain peripheral Fuc when the native polypeptide is isolated from human cerebrospinal fluid (27, 28) or from human serum (28). By contrast, no peripheral Fuc was found attached to the reverse transcription product was purified by phenol extraction, and 1 μl corresponding to 1 μg of total RNA was used in the PCR. The long form of human FT4 (6) was cloned using the sense primer 5′-CAA GAG TAG CGG ATG AGG CGC TTG and the antisense primer 5′-GCC TTC CAG TCA GTT CTG TCA in the presence of 1.3 M betaine as has been recommended for GC-rich sequences (34), using the PCR conditions: 3 min at 94 °C, 35 cycles with 15 s at 93 °C, 20 s at 49 °C, 150 s at 68 °C, increased by 4 s/cycle during the last 20 cycles, and 10 min at 68 °C. For cloning of FT7 (10, 11), the sense primer 5′-TCT CTT CTG TTC ATC CTG ATC GG (SalI site underlined) and the antisense primer 5′-TCA GGC CTG AAA CCA ACC CT were applied using the conditions: 3 min at 94 °C, 35 cycles with 15 s at 94 °C; 20 s at 45 °C, 120 s at 72 °C, increased by 10 s cycle during the last 20 cycles, and 10 min at 72 °C. The construct for FT3 was the same as used previously (22); this cDNA was originally cloned by Dr. B. Seed (Boston, MA).

All DNA fragments generated by PCR were cloned into the eukaryotic expression vectors pCR3 or pCR3.(1) (Invitrogen) according to the manufacturer’s instructions. Positive clones were identified by restriction enzyme analysis. Positive clones and the correct sequence were verified by automated sequencing of both DNA strands.

Construction of Soluble FT6 Mutants—The FT6 mutant s-FT6 encodes a human FT6 where the first 51 amino acids comprising the cytoplasmic, transmembrane, and stem region (CTS) region of the enzyme are replaced by the human IL-2 signal peptide. A first DNA fragment encoding the IL-2 signal peptide was generated by PCR using the sense primer 5′-CGG AAT TCG AGC TCG CCC GGG GAT CC, the antisense primer 5′-CGC TTC CAG TCA GTT CTG TCA in the presence of 1.3 M betaine as has been recommended for GC-rich sequences (34), using the PCR conditions: 3 min at 94 °C, 35 cycles with 15 s at 94 °C, 20 s at 45 °C, 120 s at 72 °C, increased by 10 s/cycle during the last 20 cycles, and 10 min at 72 °C. The construct for FT3 was the same as used previously (22); this cDNA was originally cloned by Dr. B. Seed (Boston, MA).

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5’-megaparamer in a second PCR together with 5 pmol of the antisense primer 5’-CTC TCA GTG GAA CCA AGC CGC TAT GC and FTS cDNA as a template under the following conditions: 3 min at 94 °C, 35 cycles with 15 s at 94 °C, 20 s at 55 °C, 15 s at 72 °C, increased by 5 cycle during the last 20 cycles, and 10 min at 72 °C. The new PCR fragment was subcloned into a pBluescript vector propagated in E. coli DH5α, and cloned using the pCR3.1 TA-cloning system as described above, generating plasmid pCRBT-FT6.

Expression of Human α1,3/4-Fucosyltransferases in BHK-21 Cells—
BHK-21 cells stably expressing human β-TP (29) were transfected with the different FT constructs by using the calcium phosphate precipitation method as described (36). In each transfection, 1.5 µg of plasmid DNA was added to 4 × 10^6 cells grown to a density of 4 × 10^5 cm^2. All vectors used contained a neomycin phosphotransferase gene driven by the SV40 promoter conferring G418 resistance to cells harboring these plasmids. Three days after transfection, the cells were subcultivated 1.5 and selected in DMEM containing 10% FBS and 1.5 g/liter G418 sulfate. Using this selection procedure, usually 100–500 clones survived and were propagated in selection medium for a further time period of 2–3 weeks. Cell culture supernatants of constantly growing monolayers were then analyzed for similar β-TP expression levels by Western blot analysis and for FT activity as described below.

SDS-PAGE and Western Blot Analysis—SDS-PAGE was performed according to Laemmli (37) using 12.5% and 3% acrylamide in the methanol, and incorporation of [14C]Fuc was determined by liquid scintillation counting of the eluate. The culture supernatants of confluently growing monolayers were usually 100–500 clones survived and were propagated in selection medium for a further time period of 2–3 weeks. Cell culture supernatants of constantly growing monolayers were then analyzed for similar β-TP expression levels by Western blot analysis and for FT activity as described below.

Production and Purification of Recombinant Human β-TP—BHK cells expressing human β-TP were grown to confluence in 175-cm² culture flasks in DMEM containing 10% FBS and where then used for the production of β-TP using medium exchanges every 2–3 days with DMEM alternating containing 0 or 2% FBS for a period of up to 3 weeks. Harvesting of rhu-β-TP was performed by Western blot analysis of cell culture supernatants at different times points during the cultivation periods and the yield for all cell lines was found to be about 1 µg of β-TP × 10^6 cells × 48 h~1. The purification of rhu-β-TP was performed by immunoaffinity chromatography using the monoclonal anti-β-TP antibody Y248D9 (39) coupled to Affi-Gel-15 matrix (Bio-Rad). In the case of BHK-21 cells expressing β-TP and BT-FT6, the latter was performed using a pBluescript vector containing the FT6 coding sequence. Immunodetection of rhu-FT6 was performed essentially as described below.

Frozen supernatants of cells transfected with different FTs was tested at 37 °C, the cells were resuspended in the absence of 0.2 mM GDP-Fuc, 100 mM NaCl, 50 mM Mops, pH 6.8, 20 mM MnCl₂, 4 mM GTP-Fuc, 0.5% Triton X-100, and 0.02% NaN₃. The protein was then precipitated with −20 °C ethanol, dried, and analyzed by MALDI/TOF-MS as described below.

Reducing N-glycans of the di- and tetraantennary complex type were incubated at 0.1–0.2 µM concentrations with 0.2 unit/liter s-FTs in the presence of 0.2 mM GDP-Fuc, 100 mM NaCl, 50 mM Mops, pH 7.5, 20 mM MnCl₂, and 0.02% NaN₃, for 24 h at 37 °C. Aliquots of the reaction mixtures were analyzed by MALDI/TOF-MS before and after desialylation and by MALDI/TOF-MS after desialylating as detailed below.

Enzymatic Release of N-Glycans Bound to Recombinant Human β-TP—Purified rhu-β-TP was reduced, carboxamidomethylated, and digested with trypsin as described previously for natural β-TP isolated from human cerebrospinal fluid (27). The tryptic glycopeptides were isolated by reversed-phase HPLC and were incubated with PNGase F as described (27). The oligosaccharides were separated from peptides by passing the incubation mixture through a reverse-phase C₁₈-column and were recovered in the flow-through.

High pH Anion-exchange Chromatography (HPAE-PAD) of Oligosaccharides—A Dionex BioLC System (Dionex, Sunnyvale, CA) equipped with a CarboPac PA1 column (4 mm × 250 mm) was used in combination with a pulsed amperometric detector (detector potentials and pulse durations: E₁ = +50 mV, T₁ = 480 s; E₂ = +500 mV, T₂ = 120 s; E₃ = −500 mV, T₃ = 60 s). The oligosaccharide material was desalted before HPAE-PAD analysis by injecting onto a Fast Desalting column (Amersham Pharmacia Biotech). In some cases, N-glycans were also desialylated prior to HPAE-PAD analysis by incubation with 0.2 unit/ml V. cholerae sialidase for 2 h at 37 °C in a buffer containing 10 mM NaOAc, pH 5.5, 1 mM CaCl₂, and 0.02% NaN₃. The oligosaccharides were then injected onto the CarboPac PA1 column that was equilibrated with 100% solvent A. Elution was performed by applying a linear gradient from 0–20% solvent B over a period of 40 min followed by a linear increase from 20–100% solvent B over 5 min. Solvent A = 0.1 M NaOH in doubly distilled H₂O, solvent B = 0.6 M NaOAc in solvent A, flow rate = 1 ml/min.

Mass Spectrometric Characterization of N-Glycans—The unseparated glycan pools obtained after enzymatic liberation from rhu-β-TP purified from the cell culture medium of the different FT cell lines were characterized after reduction and permethylation by MALDI/TOF-MS (molecular masses of all components), by ESI-MS/MS (monosaccharide sequence, presence of isomeric structures, some linkage information), and methylation analysis (substitution position). Additionally, all major carbohydrate structures detected by HPAE-PAD mapping of the native and desialylated glycans were isolated from preparative runs and were characterized by determination of the same techniques as described above.

Matrix-assisted Laser Desorption Time of Flight Mass Spectrometry—For analysis by MALDI/TOF-MS, the solutions of the reduced and permethylated oligosaccharides were mixed with the same volume of matrix (10 g/liter 2,5-dihydroxybenzoic acid in 10% ethanol in water). 1 ml of the sample was then spotted onto a stainless steel tip and dried at room temperature. The concentrations of the
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analyte mixtures were approximately 10 μm. Measurements were performed on a Bruker REFLEX™ MALDI/TOF mass spectrometer using a 357 nm (with a 3-nm pulse width and 107-108 watt/cm² irradiance at the surface (0.2 mm² spot). Spectra were recorded at an acceleration voltage of 20 kV using the reflectron and the delayed extraction facility for enhanced resolution. The following series of diantennary complex type structures bearing the proximal Fuc only or 1–2 α1,3-linked Fuc and 0–2 NeuAc residues were typically identified by their characteristic masses. Their approximate ratios were determined by assessment of the relative intensities of the respective molecular ion signals (sodium adducts, compare Fig. 1): [Hex, HexNAc]ⁿ⁺, [dHex HexNAc]ⁿ⁺, [dHex HexNAc, dHex, HexNAc]ⁿ⁺, [HexNAc, dHex, Hex, HexNAc]ⁿ⁺, etc. The linkage types present in each oligosaccharide pool were determined in this way, and particularly the degree of sialylation and the type of the Fuc residues by mild acid treatment of the respective oligosaccharides. No 3-substituted GlcNAc was detected by this procedure excluding the presence of type I motifs/Lea structures.

RESULTS

Coexpression of Human α1,3/4-Fucosyltransferases Together with Human β-TP in BHK-21 Cells—BHK-21 cells stably expressing human β-TP (29) were cotransfected with plasmids encoding human α1,3/4-FTs (FT3 to FT7) or recombinant soluble forms of FT6 (s-FT6, BT-FT6) where the catalytic domain of FT6 was fused to the C terminus of the IL-2 signal peptide or the full-length β-TP. Stably transfected cells were selected in the presence of 1.5 g/liter G418 sulfate and were used for subsequent production and characterization of the secreted β-TP. The α1,3/4-FT activity was analyzed using Gal(β1–4)GlcNAc-Oct or the type I derivative Gal(β1–3)GlcNAc-Oct in the case of FT3 as acceptors. β-TP expression levels as well as that of the BT-FT6 fusion protein were calculated from Western blot analyses of the supernatants using anti-β-TP antibodies. All stable cell lines secreted about 1 μg of recombinant β-TP/10⁶ cells/48 h in serum-free medium. For characterization of N-linked oligosaccharides of β-TP, 1–2 liters of supernatant were produced from confluent growing cells and β-TP was purified by immunoaffinity chromatography (27, 29) with a final yield of >90% as described under “Experimental Procedures.”

In Vitro and in Vivo Synthesis of Lea and sLea Motifs in β-TP N-Glycans by Recombinant Human FT6—Human β-TP expressed from BHK-21 cells in the absence of recombinant FTs contains almost exclusively diantennary complex-type II oligosaccharides at its two N-glycosylation sites. A complete structural characterization of β-TP oligosaccharides has been published elsewhere (29). About 90% of the oligosaccharides are mono- or disialylated with exclusively α1,3-linked NeuAc, and all glycans are completely proximally fucosylated in α1,6-linkage (compare molecular ion signals detected by MALDI/TOF-MS after reduction and permethylation; Fig. 1A).

When recombinant β-TP is incubated with purified s-FT6 expressed from BHK-21 cells in the presence of GDP-Fuc, three novel molecular ion peaks are detected in the MALDI-spectrum of the liberated oligosaccharides (Fig. 1B), indicating efficient peripheral fucosylation of the β-TP glycoprotein with one or two α1,3-linked Fuc resulting in Lea and sLea motifs as determined by methylation analysis (detection of the 3,4-disubstituted GlcNAc-derivative, which was not detected in oligosaccharides from wild-type β-TP, data not shown). As calculated from the peak areas of the native oligosaccharides obtained by HPAE-PAD mapping (see below), 46% of the α1,3-mono- or disialylated oligosaccharides acquired one additional α1,3-linked Fuc, whereas 33% and 6% of the α2,3-disialylated oligosaccharides were modified with one or two α1,3-linked Fuc residues, respectively. This result is in accordance with published reports for the in vitro specificity of rHuFT6, which indicate that the enzyme can form Lea as well as sLea motifs with small type II oligosaccharides (16, 21). For β-TP N-glycans isolated from the supernatant, N-glycans stably transfected with human FT6, a MALDI spectrum was obtained that differed from that of the in vivo modified β-TP N-glycans (Fig. 1C). The β-TP N-glycans, the substructure pattern of all monosaccharide constituents was determined, and in combination with the ESI-MSMS data, a detailed structural characterization of all major components was achieved. An unequivocal discrimination between the Lea and Lea structural motifs, which both yield the 3,4-disubstituted GlcNAc derivative, was performed by methylation mapping of the Fuc residues by mild acid treatment of the respective oligosaccharides. No 3-substituted GlcNAc was detected by this procedure excluding the presence of type I motifs/Lea structures.
and permethylated diantennary chains containing three Fuc or containing one Fuc, one NeuAc, and lacking one CH₂ group due to undermethylation, and furthermore MALDI/TOF-MS does not allow absolute quantification of signal intensities of different molecular ions, native oligosaccharide mixtures were also subjected to HPAE-PAD mapping, as exemplified in Fig. 2. This HPAE-PAD mapping procedure allows the separation of all possible peripherally fucosylated asialo, mono and disialo diantennary oligosaccharides that could evolve from in vitro or in vivo Fuc transfer onto the type II β-TP N-glycans except for the structure at m/z 2797, which comprises the monosialylated isomers with α,3-linked NeuAc attached to a Leα or a nonfucosylated α-N-acetyllactosamine branch. These two structures, however, can be distinguished by the application of an ESI-MS/MS technique as described under “Experimental Procedures.” The almost identical PAD responses for all structures therefore allows accurate quantification by peak integration. About 50% of the β-TP oligosaccharides contain α,1,3-linked Fuc when the protein is expressed from FT6 cells and the sialylation degree is lower when compared with structures of β-TP from cells without FT6. Most of the oligosaccharides are found to be modified with two peripheral Fuc. The ratio of SLexα-Leα antennae in the total N-glycan mixture is 1.1:1. The lower degree of sialylation of β-TP from FT6 cells could result from in vivo competition of the recombinant FT6 with the endogenous α,2,3-STs for the common asialo oligosaccharide substrate, but obviously, as is the case for in vitro incubation conditions, the enzyme can act also in vivo on both α,2,3-sialylated as well as unsialylated N-linked oligosaccharides.

When purified s-FT6 was analyzed for in vivo substrate specificity toward free complex-type N-glycans at 0.1–0.2 μM acceptor concentration (compare Table I), for both the asialo diantennary and tetraantennary type II N-glycans, only 20% of the substrate was α,1,3-mono-β-fucosylated, whereas with the α,2,3-disialylated diantennary glycan, 90% conversion to products containing one or two SLex motifs in an almost identical ratio was observed. The α,2,6-sialylated diantennary N-glycan was not recognized as a substrate. About 60% of an α,2,3-tetrasialylated tetraantennary structure was α,1,3-fucosylated, with 20% of the product containing two SLex antennae.

**Soluble Forms of FT6 Do Not Contribute to the in Vivo Specificity of the Enzyme—**As we have previously reported for the wild-type Golgi form of human FT3 expressed from BHK-21 cells (22), the full-length Golgi form of human FT6 is also subjected to considerable intracellular proteolysis, and secreted forms of the enzyme (termed sec-FT6) can be isolated from the medium of stably transfected cells. As shown in Fig. 3, sec-FT6 produces a diffuse band in SDS-PAGE with an apparent mass of about 50 kDa, whereas incubation with PNGase F reduces the apparent mass by generating two closely spaced sec-FT6 bands of about 40 kDa. Further analysis of sec-FT6 reveals only slight sensitivity to sialidase treatment, but partial resistance to Endo H treatment, indicating that in sec-FT6 most likely all four N-glycosylation sites of FT6 are present and occupied by a mixture of oligomannosidic and complex/hybrid-type N-glycans.

About 75% of the total FT6 activity was found in the supernatant of confluent FT6 cells after 48 h. In order to rule out the possibility that this proteolytic activity is a property of our host cell line, we compared a second BHK cell line (BHK-21A, see Ref. 29) and CHO DHFR− cells after transfection with human wild-type FT6. As shown in Table II, all FT6 cells showed similar expression values and similar ratios of cell-associated and cell supernatant FT activities. Therefore, the susceptibility of Golgi forms of glycosyltransferases toward intracellular proteolysis seems to be a general phenomenon, since similar observations have been reported for other recombinant glycosyltransferases by several groups (22, 42–46). However, since it was not clear to what extent intracellularly cleaved sec-FT6 contributes to the overall in vivo modification of β-TP N-glycans, we generated β-TP-secreting cell lines, which coexpress the recombinant soluble FT6 variants s-FT6 and BT-FT6 (see above). A comparison of intracellular FT6 activity and FT6 activity measured after 48 h in supernatants of confluent s-FT6 cells and BT-FT6 cells is also included in Table II. We isolated the secreted β-TP from the supernatants of all cell lines and compared the fucosylation of the diantennary oligosaccharides liberated from the purified glycoprotein. The HPAE-PAD elution profiles of the desialylated oligosaccharides are shown in Fig. 4. FT6 cells synthesize β-TP oligosaccharides with no, one or two peripheral Fuc residues in a ratio of 5:1:4 (Fig. 4A). The s-FT6(I) cell line expresses a total FT6 activity comparable to FT6 cells (cf. Table II), but no Leα-containing oligosaccharides were detected in the β-TP secreted thereof (Fig. 4B), whereas in the case of s-FT6(II) cells that express a 20-fold higher FT6 activity, small amounts of the α,1,3-mono-β-fucosylated (10%) and α,1,3-difucosylated structure (7%) were observed (Fig. 4C). The cell line expressing the chimeric BT-FT6 construct had a 80-fold higher total FT6 activity, and in this case, total β-TP oligosaccharides contained about 12% of the α,1,3-monofucosylated and 12% of the α,1,3-difucosylated diantennary structure (Fig. 4D). This result let us conclude that soluble forms of human FT6 including those generated by intracellular proteolysis of the full-length Golgi enzyme do not contribute significantly to the in vivo fucosylation properties of stably transfected FT6 cells toward secreted glycoproteins. A similar situation should also be expected in natural cells/tissues, which usually express about 50-fold lower total FT activity when compared with the recombinant BHK-21 cells (as is the case for HL-60 cells).

**Stable Coexpression of FT3, FT4, FT5, or FT7 from BHK-21**
Cells Together with $\beta$-TP—Since we have corroborated differences in in vivo and in vitro specificity of rhu-FT6, it is conceivable that such differences also exist for other human $\alpha_{1,3}$/$\alpha_{1,4}$-FTs. We therefore have constructed and selected stable cell lines expressing human FT3, FT4, FT5, and FT7 together with human $\beta$-TP as a secretory reporter glycoprotein. After purification of $\beta$-TP from each culture supernatant as described for $\alpha_{1,3}$-linked Fuc—[\textit{FT6 (CHO-DHFR)}]) prior to SDS-PAGE and Western blot analysis by using a rabbit anti-FT6-peptide antiserum. In each case, sec-FT6 purified from 15 ml culture supernatant was applied.

**FIG. 3.** Characterization of secreted forms of wild-type rhu-FT6 (sec-FT6) generated by intracellular proteolysis. Sec-FT6 was partially purified from the culture supernatant of FT6 cells by GDP-Fractogel affinity chromatography and was not treated (lane 1), or was treated with sialidase (lane 2), Endo H (lane 3), or PNGase F (lane 4) prior to SDS-PAGE and Western blot analysis by using a rabbit anti-FT6-peptide antiserum. In each case, sec-FT6 purified from 15 ml of supernatant was applied.

**TABLE I**

| Acceptor oligosaccharide | % of oligosaccharide structures containing $\alpha_{1,3}$-linked Fuc |
|--------------------------|------------------------------------------------------------|
| I. Asialo dianterennary  | 80 20                                                      |
| II. $\alpha_{2,3}$-Disialylated dianterennary | 10 45 45 — — |
| III. $\alpha_{2,6}$-Disialylated dianterennary | 100 — — — — |
| IV. Asialo tetraantennary | 80 20 — — — — |
| V. $\alpha_{2,3}$-Tetrasialylated tetraantennary | 41 47 12 Trace — |

**FIG. 2.** In vivo substrate specificity of rhu-FT6: HPAE-PAD analysis of human $\beta$-TP after coexpression in BHK-21 cells. Total $\beta$-TP N-glycans were enzymatically released from purified glycopeptides and were analyzed by HPAE-PAD mapping as detailed under “Experimental Procedures.” The structural symbols depicted are the same as used in Fig. 1. Indicated peaks were calculated to represent $\geq$90% of the total oligosaccharides of $\beta$-TP (masses were confirmed by MALDI/TOF-MS and methylation analysis as described under “Experimental Procedures”). Unidentified peaks represent either noncarbohydrate material or small amounts of triantennary structures (29) and were not considered for calculation of the results (compare also asialo oligosaccharide profiles in Fig. 4 and MALDI/TOF-MS in Fig. 1).
only a triantennary oligosaccharide containing one type I branch is modified with α,1,4-linked Fuc, although an 8 times higher type II acceptor concentration was present. Similarly, we were unable to show sLe\(^\alpha\) or Le\(^\alpha\) forming in vitro activity in extracts of FT3 cells with low molecular weight type II oligosaccharide acceptors. This finding was confirmed in a recent publication (2) describing the failure to in vitro fucosylate diantennary type II oligosaccharides with large amounts of a purified enzyme preparation. Almost no fucosylation of asialo oligosaccharides from FT3 cells, with Galβ1→3GlcNAc-Oct acceptors. Since no low molecular weight substrate was available for assessment of FT7 activity, this enzyme was measured using bovine fetuin as a substrate. A dash indicates incorporation of \(^{14}\)C]Fuc at background levels (<200 cpm corresponding to <4.0 microunits × ml\(^{-1}\) × 48 h\(^{-1}\)).

![Diagram](Image)

**Fig. 4.** In vivo functional activity of recombinant soluble forms of human FT6 coexpressed together with β-TBP in BHK-21 cells.

The diagram shows the HPAE-PAD elution profiles of β-TBP N-glycans after enzymatic desialylation. Arrows indicate the elution position of (proximally fucosylated) di-Le\(^\alpha\) diantennary and mono-Le\(^\alpha\) diantennary chains, respectively, as indicated by the symbols. Panel A, coexpression with s-FT6; panel B, coexpression with highly overexpressed s-FT6; panel C, coexpression with highly overexpressed BT-FT6 fusion protein (cf. expression levels in Table II). The BT-FT6 fusion protein was removed from the medium prior to purification of rhu-FT6 by quantitative adsorption on a GDP-Fractogel column. A minor peak marked by an asterisk represents an asialo-triantennary structure, which was not considered for the calculation of the results in this work.

**TABLE III**

**Comparison of fucosyltransferase activities and in vivo specificities of α,1,3/4-FT-transfected BHK cells**

The percentage of peripheral fucosylation of β-TBP oligosaccharides was calculated from peak areas upon HPAE-PAD mapping. FT activity was tested with Galβ1→4GlcNAc-Oct and, in the case of FT3, with Galβ1→3GlcNAc-Oct acceptors. Since no low molecular weight substrate was available for assessment of FT7 activity, this enzyme was measured using bovine fetuin as a substrate. A dash indicates incorporation of \(^{14}\)C]Fuc at background levels (<200 cpm corresponding to <4.0 microunits × ml\(^{-1}\) × 48 h\(^{-1}\)).

**TABLE IV**

**In vivo synthesis of Le\(^\alpha\) and sLe\(^\alpha\) on β-TBP N-glycans by full-length human α,1,3/4-fucosyltransferases expressed in BHK cells**

For each cell line, the secreted reporter glycoprotein β-TBP was isolated and the total oligosaccharides were characterized by MALDI/TOF-MS and were quantified by HPAE-PAD mapping as detailed for FT6 in Fig. 1 and Fig. 2. The α,1,3-fucosylated monosialo diantennary chains were found to contain exclusively Le\(^\alpha\) in the case of FT4 cells, exclusively sLe\(^\alpha\) in the case of FT7 cells, and a mixture of both isomers in the case of FT3, FT5, and FT6 cells.

**DISCUSSION**

It is difficult to confidently assess the contribution of individual FTs in the biosynthesis of specific Lewis-type carbohydrate structures as ligands for selectins in natural cells or tissues, since, based on immunohistochemical methods, in vitro enzyme activity assays as well as on RNA analyses (10, 49–52), most natural tissues or cells express more than a single FT species. This is even more difficult when considering the complex in vivo situation with different potential acceptor substrates that are presumably recognized in a different physiological environment inside the cell, e.g. glycolipids, N-, O-, or N-O-glycosylated, membrane-anchored, or secreted glycoproteins, which furthermore might be present in variable amounts.

Although most of the studies published so far on the recombinant expression of human α,1,3/4-FTs have focused on the modification of acceptors on the surface of host cells (6–10, 19, 47, 53–59) that have been discussed in context with selectin binding studies, little is known about the specificity of human α,1,3/4-FTs in the recognition of polypeptide-linked N-glycans. In the present report, we have been concentrating on the in vitro functional activity and the in vivo substrate specificity of the five different human α,1,3/4-FTs toward the N-glycans of coexpressed and secreted human β-TBP. Human β-TBP is almost exclusively decorated with diantennary complex-type II N-glycans with proximal α,6-linked Fuc when expressed from BHK-21 cells (29), the ratio of α,2,3-di-, monosialylated and asialo structures being roughly 70:25:5. The protein from nat-
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Fig. 5. ESI daughter ion mass spectrum of a reduced and permethylated monosialylated difucosylated di-antennary oligosaccharide isolated from β-TP coexpressed in FT4 cells. The monosaccharide composition of the oligosaccharide was deduced from its molecular mass, and the fragment ions generated by collision induced dissociation allowed its characterization as explained in the fragmentation scheme. It should be noted that any fucosylation of the sialylated antennae of the diantennary structure can be excluded since the expected fragment ions at m/z 1021 [NeuAc Hex dHex HexNAc + Na]+, 646 [HO-Hex dHex HexNAc + Na]+, and 486 [Hex HexNAc + Na]+ and the corresponding doubly charged ions generated by the elimination of these fragments were not detected. The linkage of the peripheral Fuc to O-3 of a GlcNAc residue is indicated by the weak, but reproducible fragment ion due to the elimination of this residue which would not be observed in the case of α1,4-linked Fuc (see also under “Experimental Procedures”).

Fig. 6. Biosynthesis of sLe\(^x\) and Le\(^x\) on β-TP oligosaccharide antennae by α1,3/4-FT-cotransfected BHK cells. The amount of β-TP N-glycan antennae containing the sLe\(^x\) or the Le\(^x\) motif was calculated from the data shown in Table IV. Black bars, sLe\(^x\); shaded bars, Le\(^x\).

The results shown here unequivocally demonstrate for the first time that all five human α1,3/4-FTs have the capability to form the sLe\(^x\) motif with complex-type II antennary oligosaccharide isolated from β-TP coexpressed in FT4 cells. The monosaccharide composition of the oligosaccharide was deduced from its molecular mass, and the fragment ions generated by collision induced dissociation allowed its characterization as explained in the fragmentation scheme. It should be noted that any fucosylation of the sialylated antennae of the diantennary structure can be excluded since the expected fragment ions at m/z 1021 [NeuAc Hex dHex HexNAc + Na]+, 646 [HO-Hex dHex HexNAc + Na]+, and 486 [Hex HexNAc + Na]+ and the corresponding doubly charged ions generated by the elimination of these fragments were not detected. The linkage of the peripheral Fuc to O-3 of a GlcNAc residue is indicated by the weak, but reproducible fragment ion due to the elimination of this residue which would not be observed in the case of α1,4-linked Fuc (see also under “Experimental Procedures”).

According to published data, FT4 exhibits substantial enzyme activity only with asialo low molecular weight type II acceptors (15, 19, 55). Our own results employing diantennary asialo or α2,3-disialylated type II N-glycans along with sec-FT4 confirmed these findings.\(^2\) We found that 46% of the β-TP glycans from FT4 cells are modified with peripheral Fuc predominantly in the Le\(^x\) motif. However, small amounts of α1,3-difucosylated monosialo as well as α1,3-monofucosylated disialo N-glycans were detected. To our knowledge, this is the first report ascribing rhu-FT4 a role in formation of the sLe\(^x\) epitope in vivo by carbohydrate structural analysis. However, supporting the hypothesis that FT4 is preponderantly a Le\(^x\)-forming enzyme is our almost exclusive detection by ESI-MS/MS of the Le\(^x\) determinant in the α1,3-fucosyltransferase N-glycan preparations do not result from partial sialylation of β-TP in the culture medium of cells or during the one-step purification procedure employed, which enabled us to quantitatively recover the recombinant protein from cell supernatants.

Interestingly, the β-TP oligosaccharides from FT4 cells were found to be undersialylated (52% sialylated versus 48% asialo antennae), whereas in β-TP from wild-type BHK-21 cells, and also from FT7 cells, about 80% of sialylated and 20% of asialo antennae were detected. This observation is consistent with previously published data (56), where the authors reported a reduced sialylation of cell surface oligosaccharides of a single CHO clone expressing FT4. However, these authors in contrast to our findings did not detect any sLe\(^x\) but reported a small amount of VIM-2 structures to be present.

FT5 cells secrete β-TP with N-glycans containing preponderantly the sLe\(^x\) motif. De Vries et al. (15) have reported that a

\(^2\) E. Grabenhorst, M. Nimtz, and H. S. Conradt, unpublished results.
soluble form of FT5 from COS-7 cells had a high preference for desialylated glycoprotein substrates as well as asialo low molecular weight acceptors in vitro. Weston et al. (7) reported a similar substrate specificity of rhu-FT5 for N-acetyllactosamine and the α2,3-sialylated derivative in cellular extracts and showed similar binding of Leα- and sLex-specific antibodies to FT5-transfected COS-1 cells indicating similar recognition of asialo and sialo structures as substrates for this enzyme. In our expression studies, however, we found a high preference for the forming of sLeα structures (sLeα:Leα about 3:1).

Although the in vivo functional activity of FT4, FT5, and FT7 can be described by their preference for either sialylated or unsialylated acceptors, FT6 acts with no such a preference. 52% of oligosaccharides of secreted β-TP from FT6 cells contained one or two peripheral Fuc residues with an almost identical ratio of sLeα to Leα antennae (1:1:1). It should be emphasized that the majority of oligosaccharides contained two peripheral Fuc, which is not the case for the N-glycans synthesized by the other FT-transfected cells except for the α1,3-difucosylated asialo structure formed by FT4. Similar to FT4 cells, the sialylation state of β-TP from FT6 cells is decreased (65% sialylated and 35% asialo antennae). This could be interpreted by a competition between rhu-FT6 and the endogenous α2,3-STs for the common Gal(α1→3)Glcnac-R acceptor, and the FT6 in vivo activity detected here should result from at least some functional co-localization of the enzymes.

The detection of the sLeα motif in β-TP glycans after coexpression of FT3 was unexpected since all our in vitro data indicated the enzyme to act only on type I chains (22). Accordingly, no Fuc transfer onto the Gal(β1→4)Glcnac-Oct could be observed by cellular extracts of FT3 cells while the Gal(β1→3)Glcnac-Oct was an efficient substrate. Since the BHK-21 cells do not synthesize any significant Gal(β1→3)Glcnac-R chains (see Refs. 29 and 32), we cannot exclude that FT3 would recognize this structure much more efficiently than the type II chains. It is noteworthy that only 11% of the total N-acetyllactosamine antennae of β-TP N-glycans from FT3 cells were substituted with peripheral Fuc, whereas with the other FTs, 22–50% of the antennae were decorated with peripheral Fuc. This again would point to a functional role of human FT3 to act as a Leα-forming enzyme in vivo and would support our previous hypothesis.

We have reported large quantities of intracellularly proteolytically cleaved forms of human FT3 expressed from BHK-21 cells that are released into the cell supernatant (22). Similar observations have been published, e.g. for β1,4-GalNac-T (44), α1,3-GalT (45), and FT6 (46). The enzymes responsible for this proteolytical cleavage have been proposed to be cathepsin-like proteases or serine proteases, respectively. Here we confirm that rhu-FT6 is secreted by two different BHK-21 cell lines and from CHO DHFR− cells. Whereas FT7 and FT6 were found to be resistant to proteolysis, we also detected secreted forms of FT4 in supernatants of transfected cells.

Our results obtained with the coexpression of genetically engineered soluble variants of FT6 (s-FT6, BT-FT6) together with β-TP indicate that the secreted enzyme fraction does not contribute to the in vivo activity, since only after about 20-fold overexpression of s-FT6, we were able to detect small amounts of fucosylated β-TP. In a recent paper (45), Cho and Cummings found by lectin binding studies that a recombinant, soluble α1,3-GalT lacking the transmembrane and cytoplasmic domain is functionally active in vivo when expressed at slightly higher levels than the full-length form. The reason for this discrepancy is unknown; however, that truncated forms of glycosyltransferases in general do not contribute significantly to their in vivo specificity toward secreted glycoproteins is supported by our finding that a recombinant soluble form of human ST6Gal does not modify co-secreted rhu-β-TP in BHK-21 cells.

Interestingly, in addition to the very low fucosylation efficiency of the high enzyme activity expressing s-FT6(II) cell line, the fucosylation pattern of β-TP glycans was also different with a higher proportion of α1,3-monofucosylated structures observed over the α1,3-difucosylated glycans, which are the major N-glycans expressed from cells transfected with full-length FT6. This then supports the view of the importance of the CTS region not only for the in vivo function of glycosyltransferases but also for their in vivo specificity. In this context, it seems attractive to speculate that the CTS region is also involved in targeting of α1,3/4 FTs into different subcompartments of the biosynthetic glycosylation pathway of cells. The CTS region could be responsible for the localization of FT6 and FT4 to subcompartments where they can compete with endogenous ST3Gal III/IV for the same acceptor as is evident from the lower sialylation state of the β-TP N-glycans secreted from the transfected cells. This is based on the assumption that ST3Gal III/IV do not act efficiently on Leα motifs (see discussion in Refs. 10, 17, and 56). The targeting properties of the FT6 CTS region should result in an intracellular broader distribution of FT6 and its overlapping with ST3Gal III/IV. Likewise, the CTS region should direct FT5 and FT7 into a later functional compartment than the BHK cell endogenous α2,3-STs, which must provide the properly sialylated oligosaccharide precursor substrates. Since the FTs and STs should be localized in trans-Golgi/trans-Golgi network subcompartments (46, 60), we believe that it will be difficult to dissect such a spatial separation by, e.g., immunolocalization techniques.

In our opinion, the approach used here for comparison of the in vivo activities of the five human α1,3/4 FTs provides new information about the involvement of the enzymes in the sequential biosynthesis of potential ligands for selectins. The stable recombinant cell lines will also provide a convenient source for the isolation of well characterized glycoprotein preparations to be used in in vivo binding or competition assays with cells/tissues expressing selectins or specific Lewis-type ligands.

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