Localization, purification and specificity of the full-length membrane-bound form of human recombinant α 1,3/4-fucosyltransferase from BHK-21B cells

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Fucosyltransferase III [galactoside 3(4)-L-fucosyltransferase; EC 2.4.1.65] (FT3) is a Golgi type II membrane protein that catalyses the synthesis of fucosylated Lewis motifs that are associated with cell-adhesion events and are differentially expressed during cell differentiation. In the present work, the full-length membrane bound form of FT3 has been expressed in baby hamster kidney cells. The enzyme has been found in the *trans*-Golgi and *trans*-Golgi network (TGN) of the transfected cells, where it appeared as monomers and dimers, but not as oligomers with high molecular masses. Therefore oligomerization is not the basis for correct localization of FT3 in the Golgi. The enzyme has been purified, with a final yield of 2% and a total purification of 2900-fold, by DEAE-Sephadex, SP-Sephadex, GDP-Fractogel and Superdex 200 chromatography. The purified enzyme showed a

clear preference for the Gal β 3GlcNAc motif in oligosaccharides conjugated with the hydrophobic tail (CH₂)₃-NHCO-(CH₂)₅-NH-biotin. Substitution of galactose with α 2-linked fucose or α 2,3-linked *N*-acetylneuraminic acid yielded a 1.9-fold increase or a 43% decrease in activity respectively. The enzyme showed no activity towards asialofetuin, a glycoprotein containing the Gal β 3GlcNAc acceptor motif. Therefore it has been concluded that the membrane-bound form of FT3 is present in the Golgi and the TGN in an equilibrium of monomers $\leftrightarrow$ dimers, which might fucosylate glycans from glycolipids, but not from glycoproteins.

Key words: dimerization, Golgi, glycosyltransferase.

INTRODUCTION

α 1,3/4-Fucosyltransferase III (FT3) (galactoside 3(4)-L-fucosyltransferase; EC 2.4.1.65) catalyses the formation of the Lewis^a (Le^a), Lewis^x (Le^x), sialyl-Le^a (sLe^a), sialyl-Le^x (sLe^x), Lewis^b (Le^b) and Lewis^y (Le^y) determinants [1]. These determinants have been shown to be involved in various cell-to-cell interactions, such as neutrophil–endothelial cell interactions during inflammation, tumour cell–endothelial cell interactions during metastasis [2], and adhesion of pathogenic agents to their host cells [3]. In cancer, fucosyltransferases, as well as other glycosyltransferases, are highly expressed, resulting in the overexpression of the Lewis antigens on the cell-surface mucins [4]. FT3 is expressed during different developmental stages, being detected in stomach, colon, small intestine, lungs and kidney [5].

Little is known about the regulation of fucosylation, and more generally glycosylation, in the Golgi. One aspect could be related to membrane fucosyltransferase organization, and the other could be associated with post-translational modifications. Glycosyltransferases are distributed along the Golgi from the *cis* to the *trans* regions and *trans*-Golgi network (TGN), and they are committed not to a single Golgi cisterna, but their localization overlaps that from other glycosyltransferases in adjacent cisternae [6]. The transmembrane domain and flanking regions of Golgi resident proteins have been shown to be essential for Golgi localization [6–8] through interactions with specific lipid domains [7] and/or with other Golgi proteins [9]. Recently, it has been shown that glycosyltransferases from *cis* and *medial* Golgi

exhibited different behaviours from those located in the *trans*-Golgi and TGN. The former would exist as large insoluble oligomers requiring high salt concentrations for solubilization, whereas the latter would exist as monomers and dimers easily solubilized with Triton X-100 (TX-100) under a low salt concentration [10]. This would probably contribute to the regulation of their activity, as well as their correct Golgi localization.

FT3, similarly to other glycosyltransferases, is proteolytically processed in its stem region, the catalytic domain being secreted into the extracellular medium and found in body fluids and cell culture medium [11]. The biological relevance of this phenomenon has not been ascertained, but it can be inferred that the regulation of fucosylation in the Golgi results from the concerted action of both membrane and soluble forms of FT3.

In the present study, we show that FT3 is localized in the Golgi/TGN of transfected baby hamster kidney (BHK) cells, where it exists as monomers and dimers. Furthermore, the transmembrane domain of the purified enzyme abolishes its activity towards glycoprotein substrate acceptors.

EXPERIMENTAL

Materials

GDP-[¹⁴C]fucose, DEAE-Sephadex and SP-Sephadex were purchased from Amersham Pharmacia Biotech; unlabelled GDP-fucose, *N*-ethylmaleimide (NEM) and Dulbecco's modified

Abbreviations used: BHK, baby hamster kidney; CB, chromatography buffer; DMEM, Dulbecco's modified Eagle's medium; FT3, α 1,3/4-fucosyltransferase III; Le^{a/b/x/y}, Lewis^{a/b/x/y}; sLe^{a/x}, sialyl-Le^{a/x}; NEM, *N*-ethylmaleimide; TGN, *trans*-Golgi network; TRITC, tetramethylrhodamine β -isothiocyanate; TX-100, Triton X-100.

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Eagle's medium were obtained from Sigma. Fetal calf serum, penicillin/streptomycin and Geneticin were obtained from Gibco BRL. The purified antibody raised against the catalytic domain of human FT3 was a gift from Dr J. Lowe (University of Michigan Medical School, Ann Arbor, MI, U.S.A.). The substrate acceptors used had a hydrophobic tail, and were as follows: (1) Gal β 3GlcNAc-O(CH $_2$) $_8$ COOMe and Gal β 4GlcNAc-O(CH $_2$) $_8$ COOMe, which were a gift from Dr O. Hindsgaul (University of Alberta, Canada); (2) oligosaccharides conjugated to (CH $_2$) $_3$ -NHCO-(CH $_2$) $_5$ -NH-biotin (Syntesome, Munich, Germany). Gal β 4GlcNAc-1,2-di-O-hexadecyl-*sn*-glycero-3-phosphoethanolamine was from Dextra (Reading, Herts., U.K.). All other chemicals were obtained from other commercial sources and were of the highest purity available.

Cell culture

Wild-type BHK cells and transfected BHK cells stably expressing wild-type FT3 (BHK-FT3) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 1 mg/ml Geneticin sulphate for transfected cells. BHK-FT3 cells were obtained by transfection of BHK cells with the pCRFT3 vector by the calcium phosphate precipitation method and selection with 1 mg/ml Geneticin sulphate. Cells were grown in a humidified incubator at 37 °C in an atmosphere of 5% CO $_2$.

Vector construction

The vector pCRFT3 expressing the full-length wild-type form of FT3 was generated by PCR-based site-directed mutagenesis, using the previously described vector pCRFT3T2 as the template, to remove the tag coding an epitope derived from the cytomegalovirus glycoprotein ICP36 [11]. Primers used for the PCR reaction were N-terminus primer (reading from 5' to 3') TGACCCATGGATCCCCCTGGGTGCAGCCAAG, and C-terminus primer (from 5' to 3') GGCCTCTCAGGTGAACCAAGCCGCTATGCT. PCR amplification was performed in a reaction mixture containing 1 μ g/ml of template DNA, 1 μ M of each primer, 1 μ M dNTPs, 6% (v/v) DMSO, a suitable buffer and 2 units of *Turbo-Pfu* DNA polymerase (Stratagene). Applied PCR cycling conditions were as follows: denaturing step at 95 °C for 30 s, annealing step at 65 °C for 30 s, and extension step at 72 °C for 2 min, for 30 cycles. Amplified fragments (1.1 kb) were analysed by electrophoresis in 0.8% (w/v) agarose gels, and purified. The resulting purified DNA fragments were directly ligated to pCR3.1-uni vector to obtain the pCRFT3 vector, and the sequence was confirmed by DNA sequencing. General techniques for clone analysis and maintenance were performed by standard techniques.

Immunofluorescence microscopy

Half-confluent cells grown on glass coverslips were washed with PBS containing 0.5 mM MgCl $_2$, fixed with 3% (w/v) para-formaldehyde in PBS for 30 min, and permeabilized with 0.1% (w/v) TX-100 for 15 min. Fixed cells were blocked with 1% (w/v) BSA in PBS for 1 h, and incubated for 2 and 1 h with primary and secondary antibodies respectively. Antibodies were diluted in PBS containing 1% (w/v) BSA, and washes were done with PBS. Coverslips were mounted in Airvol and examined under a Bio-Rad MRC1024 confocal microscope or a Leica DMRB microscope. The following antibodies in the dilutions indicated were used: monoclonal mouse anti-Golgi 58K protein (Sigma), 1:50; mouse monoclonal anti-(AP-1) and

anti-(AP-2) (β_1 and β_2 adaptins respectively; Sigma), 1:500. Rabbit antiserum anti-FT3 obtained from Dr John Lowe was used at a dilution of 1:20 for permeabilized, or of 1:50 for non-permeabilized, cells respectively; the secondary antibodies (Sigma) included goat anti-(mouse IgG)-tetramethylrhodamine β -isothiocyanate (TRITC) conjugate at a dilution of 1:64, and goat anti-(rabbit IgG)-FITC conjugate diluted 1:160. Where indicated, cells were incubated with media containing 5 μ g/ml brefeldin A, 10 μ M nocodazole (both for 45 min) or 100 or 750 μ g/ml cycloheximide for 4 h at 37 °C before fixation.

FT3 solubilization

BHK-FT3 cells grown to confluency were resuspended in MNT buffer [25 mM Mes (pH 6.5)/1% (w/v) TX-100] containing NaCl concentrations from 0 to 250 mM in the presence or absence of 30 mM NEM. The following protease inhibitors were added to the MNT buffer: 0.2 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride ('AEBSF'), 1.0 mM EDTA, 1 μ g/ml aprotinin, 10 μ g/ml leupeptin and 10 μ g/ml pepstatin A. The extraction took place at 4 °C for 1 h and the extracts were centrifuged for 15 min at 15000 *g*. The supernatant was centrifuged further for 1 h at 100000 *g*. The pellets and the supernatant were precipitated with ethanol, and analysed by SDS/PAGE followed by Western blotting.

FT3 purification

BHK-FT3 cells were pelleted at 800 *g* for 5 min at 4 °C, resuspended in 4 volumes of homogenization buffer consisting of 20 mM Mes/NaOH, pH 6.8, 130 mM NaCl containing the protease inhibitors indicated above, and homogenized on a Potter-Elvehjem with 20 strokes on ice. Nuclei were separated by centrifugation at 1000 *g* for 10 min at 4 °C, and the supernatant was centrifuged at 100000 *g* and 4 °C for 1 h. The mitochondria- and microsomes-containing pellet was resuspended in extraction buffer [20 mM Mes/NaOH (pH 6.8)/100 mM NaCl/1% (w/v) TX-100/25% (w/v) glycerol/1 mM dithiotreitol/0.02% (w/v) sodium azide]. Extraction took place for 15 h at 4 °C under gentle stirring. The mixture was centrifuged at 100000 *g* for 1 h at 4 °C, and the supernatant collected as the TX-100 extract. All purification steps were performed in chromatography buffer (CB) [20 mM Mes/NaOH/1% (w/v) TX-100/25% (w/v) glycerol/1 mM dithiotreitol/0.02% (w/v) sodium azide], with the indicated variations in the pH and NaCl concentration.

DEAE-Sepharose batch

The TX-100 extract was adjusted to pH 6.0 and mixed with DEAE-Sepharose previously washed and equilibrated with CB at pH 6.0 containing 100 mM NaCl. The mixture was left at 4 °C for 2 h under gentle stirring, centrifuged at 5000 *g* for 5 min, and the active supernatant collected for further purification steps. The bound proteins were eluted with CB at pH 6.2 containing 1 M NaCl.

SP-Sepharose chromatography

The supernatant obtained from the DEAE-Sepharose batch was adjusted to pH 6.2 with 1.0 M NaOH, and loaded at 0.2 ml/min on an SP-Sepharose column (4 cm $\times$ 1 cm) previously equilibrated with CB at pH 6.2 containing 100 mM NaCl. The activity was eluted with 2.5 bed-volumes of a linear gradient from 100 to 500 mM NaCl in CB.

GDP–Fractogel affinity chromatographies

Activity-containing fractions from SP-Sepharose were loaded at 0.5 ml/min on to a GDP–Fractogel column (2.5 cm $\times$ 1 cm) previously equilibrated with CB at pH 6.2 containing 500 mM NaCl buffer (GDP–Fractogel 1). The activity was eluted with 10 volumes of a linear gradient of 0.5 to 1.0 M NaCl in CB at 1.5 ml/min. The active fractions were pooled, adjusted to 0.5 M NaCl by dilution with CB at pH 6.2, and loaded on to the same column previously equilibrated with the same buffer (GDP–Fractogel 2). The activity was eluted with a linear gradient from 0 to 100 μ M GDP.

Superdex 200 gel filtration

The active fractions from the GDP gradient were concentrated and loaded at 0.1 ml/min on to a Superdex column (22 cm $\times$ 1.5 cm) previously equilibrated with CB at pH 6.2 containing 500 mM NaCl. The column was calibrated with the gel-filtration molecular markers Dextran Blue, thyroglobulin (669 kDa), ferritin (440 kDa), alcohol dehydrogenase (150 kDa), BSA (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (29 kDa) in CB with 500 mM NaCl at 0.1 ml/min. Fractions were analysed for fucosyltransferase activity with the Gal β 3GlcNAc-O(CH₂)₈COOMe acceptor or by Western blotting, as described below.

SDS/PAGE and Western blot analysis

Samples were precipitated with 8% (w/v) trichloroacetic acid, resuspended in the presence or absence of 5% (w/v) 2-mercaptoethanol, and analysed by SDS/PAGE [12] and Western blotting as described previously [13]. Protein concentration was determined by the bicinchoninic acid method [14].

Enzyme activity assay

The enzyme activity was measured by the incorporation of [¹⁴C]fucose from GDP-[¹⁴C]fucose into several acceptors as described previously [13]. The acceptors were as follows: Gal β 3GlcNAc-O(CH₂)₈COOMe for enzyme purification; Gal β 3GlcNAc-R, Fuc α 2Gal β 3GlcNAc-R, NeuAc α 2,3Gal β 3GlcNAc-R, Gal β 4GlcNAc-R, Fuc α 2Gal β 4GlcNAc-R, NeuAc α 2,3Gal β 4GlcNAc-R, where R = -(CH₂)₅-NHCO-(CH₂)₅-NH-biotin, asialofetuin and Gal β 4GlcNAc-1,2-di-O-hexadecyl-*sn*-glycero-3-phosphoethanolamine at 1.7 mM for specificity studies. Asialofetuin (4 mg/ml) and the glycolipid (1.7 mM) were assayed for 3 h.

RESULTS AND DISCUSSION

Human FT3 is localized in the Golgi/TGN of transfected BHK cells

BHK cells were transfected with the plasmid pCRFT3 that contained the full-length membrane-bound form of FT3. No endogenous FT3 was detected on wild-type BHK cells by enzyme activity measurement or immunofluorescence microscopy. FT3 was detected in the periphery of the nucleus, and it was found to co-localize with the 58 K protein, a commonly used Golgi marker (Figures 1A and 1B) [15]. When the cells were incubated with anti-(AP-1) and anti-(AP-2) protein complexes, partial co-localization with FT3 was also observed (Figures 1B and 1C). The co-localization was restricted to a perinuclear region corresponding to the TGN, where AP-1 is localized [16]. The antibody anti-(AP-1)/anti-(AP-2) stained structures different from the TGN, corresponding to AP-2 on the plasma membrane

and endocytic vesicles [17]. Incubation with 750 μ g/ml cycloheximide did not cause alterations in the fluorescence (Figure 1E), whereas for a cell line expressing the secreted form of FT3, SFT3 (previously described as S2FT3T2; [11]), Golgi-associated fluorescence disappeared after incubation with cycloheximide (Figures 1F and 1G), whereas large vesicles, probably corresponding to dilated endoplasmic reticulum, persisted [18]. Incubation of BHK-FT3 cells with brefeldin A caused a dispersed reticular pattern characteristic of the endoplasmic reticulum for FT3 (Figure 1H). After incubation with nocodazole, the fluorescence was found partly in vesicles dispersed in the cytoplasm (Figure 1I). Taken together, these results indicate that the enzyme was distributed within the *trans*-Golgi and TGN. Furthermore, it has been shown previously that the enzyme was active in the Golgi of BHK cells, where it catalysed the synthesis of the sLe^x motif [19]. This result supported the presence of FT3 in the Golgi/TGN, because it uses as an acceptor substrate a sialylated biantennary glycan, and β 4-galactosyltransferase has been found in the *trans*-Golgi/TGN [20]. FT3 was not detected on the cell surface of non-permeabilized cells (Figure 1G).

Oligomerization of FT3

In order to investigate the oligomerization state of FT3 in the *trans*-Golgi and TGN, BHK-FT3 cells were extracted with 1% (w/v) TX-100 with increasing NaCl concentrations, the extracts were successively centrifuged at 15000 *g* and 100000 *g* as described by Opat et al. [10]. Extraction was performed at pH 6.5, which is the pH described for the Golgi [21–23]. The pellets and supernatants were analysed by Western blot analysis, and it was observed that FT3 was almost totally solubilized with 100 mM NaCl (Figure 2). This behaviour closely resembled that described for the *trans*-Golgi and TGN β 4-galactosyltransferase and α 2-fucosyltransferase, which were solubilized with 50–100 mM NaCl. In contrast, *cis*- and *medial*-Golgi enzymes such as mannosidase II and N-acetylglucosaminyltransferases I and II exist as large protein complexes that require 200–250 mM NaCl for complete solubilization [10,24–26].

On the other hand, dimerization of several late-acting glycosyltransferases has been observed [27–30]. In order to investigate FT3 dimerization, BHK-FT3 cells were extracted as described above, in the absence or presence of 30 mM NEM, and analysed by non-reducing SDS/PAGE and Western blotting (Figure 3). It was observed that FT3 appeared as a monomer + dimer (Figure 3A). For 0 mM NaCl, solubilized FT3 appeared mostly as a monomer. Increasing the salt concentration to 100 and 200 mM NaCl caused total solubilization of monomer + dimer. When the extractions were performed in the presence of NEM in order to block all reactive SH groups and prevent post-homogenization dimer formation, the presence of solubilized monomer + dimer was also observed (Figure 3B). The dimer was not detected when the 200 mM extract was analysed by reducing SDS/PAGE, confirming that the dimer formation was mediated by disulphide bonds (Figure 3A). Therefore, it was concluded that FT3 existed in the Golgi membranes in an equilibrium of monomer $\leftrightarrow$ dimer, as previously observed for other late-acting Golgi enzymes such as β 4-galactosyltransferase [27] and α 2,6-sialyltransferase [28] from transfected COS cells, and fucosyltransferase VI [29] or α 2,8-sialyltransferase [30] from transfected CHO cells.

The mechanisms underlying the correct localization of glycosyltransferases in the Golgi are unknown at present. It has been proposed that the formation of large homo- or hetero-oligomers might correlate with Golgi localization [24]. Since these oligomers do not exist for FT3, similarly to other *trans*-Golgi

Figure 1 Indirect immunofluorescence microscopy of stably transfected BHK-FT3 cells

FT3 (**A, C, E, H–J**) and SFT3 (**F, G**) were detected with an antiserum raised against the catalytic domain of FT3, and as a secondary antibody an anti-(rabbit IgG) labelled with FITC was used in cells fixed with 3% (v/v) paraformaldehyde and permeabilized with 0.1% (w/v) TX-100 (**A–I**), or in non-permeabilized cells (**J**). Co-localization of FT3 (**A, C**) with the Golgi 58K protein (**B**) detected with an anti-58 K monoclonal antibody, or with the AP-1 and AP-2 protein complexes (**D**) detected with an anti-AP-1 and AP-2 monoclonal antibody. Anti-(mouse IgG) secondary antibody was labelled with TRITC. (**E**) and (**F**) Effect of 750 µg/ml cycloheximide on BHK-FT3 and BHK-SFT3 cells compared with untreated cells (**A** and **G** respectively). (**H**) and (**I**) Effect of 5 µg/ml brefeldin A and 10 µM nocodazole on BHK-FT3 cells. The cells were photographed for FITC (**A, C, E–J**) or TRITC (**B, D**) signals on a Bio-Rad MRC1024 confocal (**A–D**) or a Leica DMRB (**E–I**) microscope. Bar = 10 µm.

Figure 2 FT3 solubilization with different NaCl concentrations

BHK-FT3 cells were extracted with 25 mM Mes, pH 6.5, containing 1% (w/v) TX-100 and different NaCl concentrations (0, 50, 100, 150, 200, and 250 mM). The extracts were centrifuged for 15 min at 15 000 *g* and the supernatant was further centrifuged at 100 000 *g* for 1 h. The 15 000 *g* pellet (P1) and the 100 000 *g* pellet (P2) were made to the same volume as the final supernatant (S) and were analysed by SDS/PAGE and Western blotting as described in the Experimental section.

Figure 3 FT3 dimerization

BHK-FT3 cells were extracted as indicated in the legend to Figure 2, in the absence (**A**) or presence (**B**) of 30 mM NEM. The extracts were analysed under non-reducing or reducing conditions (SR) by SDS/PAGE, and FT3 was detected by Western blot. Monomer and dimer are indicated by arrows on the right. Non-specific ligation from BHK cells is indicated by an arrowhead.

and TGN glycosyltransferases, it is likely that another type of mechanism would be responsible for the correct localization of these Golgi resident proteins. In this case, properties of the transmembrane domain, such as the length [31,32] and the presence of dibasic motifs in the cytosolic domain [33], could contribute to Golgi localization. These domains could mediate interactions with proteins responsible for the concentration of glycosyltransferases into retrograde COP I vesicles [9], accounting for the maintenance of steady-state composition of Golgi enzymes according to the cisternal maturation model.

FT3 purification

Frozen BHK-FT3 cells were homogenized in a Potter–Elvehjem homogenizer, and the microsomal fraction was solubilized with 1% (w/v) TX-100. Most of the enzyme activity (72%) was recovered from the cell homogenate in the TX-100-soluble

fraction, with 5% of the total activity remaining in the insoluble pellet. The amount of total FT3 detected by Western blotting in the insoluble pellet was also very low. These results confirmed that FT3 did not form large oligomers at this pH value, in contrast with that observed for another Golgi/TGN glycosyltransferase, human α 2,6-sialyltransferase, expressed in transfected COS-1 cells [28]. The TX-100 extract was added to DEAE-Sepharose with the majority of α 1–4 enzyme activity remaining in the supernatant. The supernatant was adjusted to pH 6.2 and loaded on to an SP-Sepharose column, and the enzyme was eluted with 0.5 M NaCl (Figure 4A). In this step, 44% of the loaded activity was recovered (Table 1). The active fractions were then loaded on a GDP–Fractogel column and eluted with a linear NaCl gradient. The activity peak was obtained at 0.8 M NaCl (Figure 4B) with 50% enzyme activity recovery and 5.3-fold purification.

The enzyme was loaded further on the GDP–Fractogel and eluted at 70 μ M GDP with a linear GDP gradient. The fractions were analysed by Western blot analysis, and the enzyme-containing fractions were concentrated and loaded on to a Superdex 200 column. In the final step, FT3 was separated in a Superdex 200 column, and appeared as a broad peak composed of overlapping peaks with approximate masses of 213, 112 and 50 kDa (Figure 4C). The analysis of this peak by SDS/PAGE showed that FT3 was eluted as a single major band of 45 kDa (Figure 4D). This was the expected molecular mass for the full-length FT3 (42 069 Da) [1] with two occupied glycosylation sites (increase of approx. 3–4 kDa). FT3 was purified $\approx$ 2900-fold with 2% yield from the starting homogenate. The molecular mass overestimation of FT3 in gel filtration was possibly due to detergent association, because it is known that polyethylene glycol detergents used for protein solubilization alter the hydrodynamic volume of proteins, yielding an overestimation of their mass associated with a relatively early elution on gel-chromatography columns [34]. However, since it is known that TX-100 has the lowest binding capacity for proteins [35], the overestimation could also be due to protein association, similar to that found for N-acetylglucosaminyltransferase II [36]. In order to investigate the dimerization of FT3 eluting from the Superdex column, fractions were analysed by non-reducing SDS/PAGE and Western blotting. The formation of a disulphide-bonded homodimer was observed, and the ratio of monomer to dimer was higher for later-eluting fractions (Figure 4E). Thus the enzyme specificity observed was due to a mixture of monomer+dimer of FT3, similar to that found for the enzyme present in the Golgi membranes.

Enzyme specificity

The acceptor specificities of the full-length FT3 from transfected BHK cells towards type 1 Gal β 1–3GlcNAc (compounds **1** to **3**, Table 2) and type 2 Gal β 1–4GlcNAc (**4** to **6** Table 2) acceptors

Figure 4 Purification of FT3

(A) Cation exchange on SP-Sepharose. The NaCl gradient (0–500 mM) is indicated by a broken line (axis not shown); **(B)** Affinity on GDP-Fractogel1. NaCl gradient from 0.5 to 1.5 M; **(C)** Gel filtration on Superdex 200. The elution of standards (molecular mass in kDa) is shown on the top of the panel. **(D)** SDS/PAGE analysis of the purification steps. Lane 1, TX-100 extract; lane 2, NaCl elution from the GDP–Fractogel; lane 3, broad peak from the Superdex 200 column. Protein concentration (●) and α 1,4-fucosyltransferase activity (■) are described in the Experimental section. **(E)** FT3 eluting at 9.6 and 11.0 ml (lanes 1 and 2 respectively) obtained from the Superdex 200 gel filtration was analysed by non-reducing SDS/PAGE and detected by Western blotting.

Table 1 Purification of human fucosyltransferase III from transfected BHK cells

One unit corresponds to 1 μ mol of fucose/min transferred under the conditions of the assay described in the Experimental section.

Step	Total protein (mg)	Total volume (ml)	Total activity (units $\times 10^{-3}$)	Specific activity (units $\times 10^{-3}$ /mg)	Step purification (fold)	Total purification (fold)	Step yield (%)	Total yield (%)
Homogenate	99.41	15.3	5.74	0.0577	1	1	100	100
TX-100 extraction	22.95	15.3	4.13	0.180	3.13	3	72	72
DEAE-Sepharose	16.01	15.3	2.89	0.181	1	3	70	50
SP-Sepharose	1.32	12.3	1.27	0.963	5.35	17	44	22
GDP-Fractogel1 (NaCl Elution)	0.2825	7.5	0.713	2.52	2.61	44	56	12
GDP-Fractogel2 + Superdex 200	0.00069	3	0.114	165.2	65.5	2863	16	2

were studied [37]. Similarly to the soluble secreted form of FT3 from BHK cells [11], it was observed that FT3 has a clear preference for the type 1 acceptor (**1**). Substitution of the galactose with α 2-linked fucose (**2**) yielded a 1.9-fold increase in activity, whereas substitution of the galactose with α 2,3-linked

N-acetylneuraminic acid (**3**) yielded a 43 % reduction in activity. There was no activity detected with non-sialylated (**4**) and sialylated (**6**) type 2 substrates, whereas α 2-fucosylated type 2 acceptor was a very poor substrate for the enzyme. These results essentially agreed with those previously reported for crude

Table 2 Substrate specificity of FT3 from transfected BHK cells

Activity is relative to Gal β 3GlcNAc-R at 1.7 mM concentration in the presence of 0.05 mM GDP-fucose; R = $-(CH_2)_3-NHCO-(CH_2)_2-NH$ -biotin. ND, not detected.

Substrate	Activity (m-units/ml)	Relative activity (%)
Gal β 1,3GlcNAc-R (1)	1.4 (2.6)*	100 (100)*
Fuc α 1,2Gal β 1,3GlcNAc-R (2)	2.7	193
NeuAc α 2,3Gal β 1,3GlcNAc-R (3)	0.8	57
Gal β 1,4GlcNAc-R (4)	ND	ND
Fuc α 1,2Gal β 1,4GlcNAc-R (5)	0.13	9.3
NeuAc α 2,3Gal β 1,4GlcNAc-R (6)	ND	ND
Asialofetuin	ND (0.2)*	ND (7.7)*

* Values between brackets refer to the activity observed using the soluble form of FT3 (SFT3) as the enzyme.

cellular extracts containing full-length FT3 linked to protein A [38]. Another acceptor tested was the glycoprotein asialofetuin, which contains biantennary (two peripheral type 2 determinants), triantennary 2,4-branched (three peripheral type 2 determinants) and triantennary 2,4-branched (two peripheral type 2 determinants and one peripheral type 1 determinant) in a proportion of 10:55:35 [11]. FT3 showed no detectable activity towards the glycoprotein asialofetuin in the presence of TX-100 (Table 2). In contrast, the corresponding soluble form of the enzyme, SFT3, with the N-terminus located at Ala⁴⁷, uses this glycoprotein as substrate, as previously reported (Table 2, values in parentheses) [11]. Even when the time of the reaction was extended for 3 h and the ratio of labelled to non-labelled GDP-Fuc was increased by 3-fold, FT3 did not recognize asialofetuin as an acceptor. The lack of activity of FT3 towards asialofetuin was not due to the presence of the detergent, because we have observed that 1% TX-100 caused a 70% activation of SFT3 towards asialofetuin. Thus it was concluded that the presence of the 46 residues from the N-terminus prevented or greatly decreased activity of FT3 towards glycoprotein acceptors. Similar results have been reported previously for crude extracts containing full-length FT3 linked to protein A [38]. Since a significant amount of fucosyltransferase activity (40%) from BHK cells transfected with FT3 is proteolytically cleaved and secreted [11], it is possible that the activity of FT3 in the Golgi of these cells is due to both the full-length and the soluble forms of the enzyme, glycoproteins being preferentially fucosylated by the proteolytically cleaved form.

Conclusions

FT3 is present in an equilibrium of monomer $\leftrightarrow$ dimer in the *trans*-Golgi/TGN of transfected BHK cells, therefore oligomerization into higher-molecular-mass complexes cannot account for either the retention of the enzyme in the Golgi or its specificity in the BHK cells. The amino acid sequence Met¹ to Arg⁴⁶ prevents the enzyme from using glycoproteins as acceptor substrates.

We thank Dr John Lowe for the gift of the FT3 antibody and Dr O. Hindsgaul for the gift of FT3 acceptors. We thank Nuno Moreno for the technical assistance with the confocal microscope. This work was funded by grants SAU/1411/1998 and BIO/12072/1998, FCT, Portugal to J.C. V.L.S. and M.T.C. were recipients of fellowships from FCT. F.E. received an EMBO fellowship.

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Received 10 January 2001/10 May 2001; accepted 4 June 2001