Perturbation of free oligosaccharide trafficking in endoplasmic reticulum glucosidase I-deficient and castanospermine-treated cells[1]

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Free oligosaccharides (FOS) are generated both in the endoplasmic reticulum (ER) and in the cytosol during glycoprotein biosynthesis. ER lumenal FOS possessing the di-*N*-acetylchitobiose moiety at their reducing termini (FOSGN2) are exported into the cytosol where they, along with their cytosolically generated counterparts possessing a single *N*-acetylglucosamine residue at their reducing termini (FOSGN1), are trimmed in order to be imported into lysosomes for final degradation. Both the ER and lysosomal FOS transport processes are unable to translocate triglucosylated FOS across membranes. In the present study, we have examined FOS trafficking in HepG2 cells treated with the glucosidase inhibitor castanospermine. We have shown that triglucosylated FOSGN2 generated in the ER are transported to the Golgi apparatus where they are deglucosylated by endomannosidase and acquire complex, sialic acid-containing structures before being secreted into the extracellular space by a Brefeldin A-sensitive pathway. FOSGN2 are also secreted from glucosidase I-deficient Lec23 cells and from the castanospermine-treated parental Chinese-hamster ovary cell line. Despite the secretion of FOSGN2 from Lec23 cells, we noted a transient intracellular accumulation (60 nmol/g cells) of triglucosylated FOSGN1 in these cells. Finally, in glucosidase I-compromised cells, FOS trafficking was severely perturbed leading to both the secretion of FOSGN2 into the extracellular space and a growth-dependent pile up of triglucosylated FOSGN1 in the cytosol. The possibility that these abnormalities contributed to the severe and rapidly progressive pathology in a patient with congenital disorders of glycosylation type IIb (glucosidase I deficiency) is discussed.

Key words: congenital disorders of glycosylation type IIb, glycoprotein biosynthesis, Golgi apparatus, secretory pathway, transport.

INTRODUCTION

Glycoproteins bearing N-linked polymannose-type oligosaccharides are synthesized in the lumen of the endoplasmic reticulum (ER) before being delivered to the Golgi apparatus where their sugar chains are remodelled to form complex *N*-glycans [1–3]. Glycoproteins bearing these complex glycans are then, mostly, secreted or delivered to the plasma membrane where their sugar chains are known to play important roles in cellular signalling [4]. Appropriate expression of glycoproteins requires that they first fold correctly and then are accurately targeted to their final destinations.

Protein glycosylation begins with the transfer of the oligosaccharide $Glc_3Man_9GlcNAc_2$ from dolichol-PP_i to nascent polypeptides in the lumen of the ER [1,2]. After rapid removal of the glucose residues by ER-situated glucosidases I and II, glycoproteins may undergo several cycles of monoglucosylation/deglucosylation during folding in the lumen of the ER [5]. This process is performed by the misfolded glycoprotein sensor, UDP-Glc:glycoprotein glucosyltransferase and glucosidase II, and it allows misfolded glycoproteins to interact transiently with the lectins calnexin and calreticulin, thereby prolonging the contact of misfolded structures with the folding apparatus [6]. The importance of the removal of the three glucose residues that were initially found on nascent glycoproteins is attested by the fact that glucosidase I and II inhibitors, such as castanospermine (CST), have profound effects on the early events during glycoprotein metabolism [7]. For example, treatment of cells with CST slows down the secretion of certain glycoproteins [8], blocks the entry of glycoproteins into the autophagic pathway [9] and stimulates a rapid degradation of some glycoproteins [10].

Glucose residues are now also known to play important roles in the trafficking and catabolism of free oligosaccharides (FOS) produced during glycoprotein biosynthesis. FOS possessing the di-*N*-acetylchitobiase moiety at their reducing termini (FOSGN2) are generated in the lumen of the ER; this is caused by the hydrolytic action of oligosaccharyltransferase on $Glc_3Man_9GlcNAc_2$-PP-dolichol [11,12] and by the action of an ER lumenal *N*-glycanase on misfolded glycoproteins [13]. After deglucosylation by ER glucosidases I and II, these structures are rapidly cleared from the lumen of the ER into the cytosol by transport machinery [14] that is unable to recognize triglucosylated FOS [15]. FOS are also generated in the cytosol during ER-associated protein degradation by the de-N-glycosylation [16,17] of misfolded glycoproteins that have been translocated out of the ER into the cytosol to be degraded by the proteasome [18,19]. Cytosolic FOSGN2 are rapidly acted upon by endo-β-*N*-acetylglucosaminidase (endo H) [20] or chitobiase [21] to yield FOS possessing a single N-acetylglucosamine residue at their reducing termini (FOSGN1), which are subsequently partially demannosylated and then imported into lysosomes to be degraded [22,23]. *In vitro* studies have demonstrated that the preferred substrates for lysosomal import are small ($Man_3GlcNAc > Man_4GlcNAc > Man_5GlcNAc \gg$

Abbreviations used: BFA, Brefeldin A; CHO, Chinese-hamster ovary; conA, concanavalin A; CST, castanospermine; endo H, endo-β-*N*-acetylglucosaminidase; ER, endoplasmic reticulum; FCS, foetal calf serum; FOS, free oligosaccharides; FOSGN1, FOS possessing a single *N*-acetylglucosamine residue at their reducing termini; FOSGN2, FOS possessing the di-*N*-acetylchitobiose moiety at their reducing termini; KIF, kifunensin; PB, permeabilization buffer; QAE, quaternary aminoethyl; SLO, streptolysin O; TCA, trichloroacetic acid.

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$Man_9GlcNAc$) FOS [23], and experiments with intact cells indicate that $Glc_3Man_{9-5}GlcNAc$ structures accumulate in the cytosol of CST-treated HepG2 cells [24].

A patient diagnosed with a deficiency of ER glucosidase I has been described recently [25]. The clinical symptoms were severe and progressed rapidly, and the child died 90 days after birth. Currently, it is not clear which ramifications of a deficiency in ER glucosidase I could generate such a dramatic clinical picture. In the present study, we have addressed the possibility that changes in the trafficking of FOS were a contributory factor to the rapid progression of this disease. Therefore we have followed the fate of triglucosylated FOS in CST-treated HepG2 cells and also in the glucosidase I-deficient Chinese-hamster ovary (CHO) cell line, Lec23 [26]. We have shown that, in glucosidase I-impaired cells, FOSGN2 remain in the secretory pathway and can be recovered from the extracellular medium after their passage through the Golgi apparatus. We proceeded to quantify the pile up of cytosolic triglucosylated FOSGN1 in Lec23 cells and to demonstrate that these cells can accumulate approx. 60 nmol of FOS/g wet cells; however, the observed accumulation was transient, indicating that these cells possess alternative routes for the clearance of FOS from the cytosol.

MATERIALS AND METHODS

Materials

Methyl-α-D-glucopyranoside, Brefeldin A (BFA) and pronase were purchased from Sigma. Benzyl α-D-mannoside was purchased from Dextra Laboratories (Reading, Berks., U.K.). $Man_9GlcNAc_2$, *Clostridium perfringens* sialidase, endo H and alkaline phosphatase were purchased from Oxford GlycoSciences (Abingdon, Berks., U.K.). CST was purchased from Cambridge Research Biochemicals (Northwich, Cheshire U.K.). Kifunensin (KIF) and methyl-α-D-mannopyranoside were purchased from Toronto Research Chemicals (Toronto, Canada). 2-Aminopyridine and sodium cyanoborohydride were obtained from Sigma–Aldrich Fine Chemicals (St. Quentin-Fallavier, France), all cell culture reagents were from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.), streptolysin O (SLO) from Difco (Biovalley, S.A., Conches, France) and [2-^{3}H]mannose from NEN Life Science Products (Zaventum, Belgium). TLC plates were obtained from Merck (Darmstadt, Germany) and concanavalin A (conA)–Sepharose from Pharmacia (Uppsala, Sweden).

Culture and radiolabelling of cells

HepG2 cells were cultivated in RPMI 1640 containing 10% foetal calf serum (FCS) as described previously [27] and pulse-radiolabelled with 100 μCi D-[2-^{3}H]mannose (20 Ci/mmol) for 20 min in 0.5 ml glucose-free RPMI 1640 medium supplemented with 5% dialysed FCS, 2 mM glutamine, 5 mM fucose and 1 mM sodium pyruvate. Fucose was added to the pulse incubations to block the incorporation of radioactive fucose into glycoproteins; increasing the intracellular concentration of GDP–fucose leads to inhibition of the conversion of GDP–[^{3}H]mannose into GDP–[^{3}H]fucose [28]. In a typical pulse incubation performed with 90% confluent cells in a 6-well tissue-culture plate, 1–4% of the radioactivity was found to be incorporated into glycoproteins, glycolipids and FOS. Chase incubations were performed in complete growth medium. When pulse–chase studies were performed in the presence of different inhibitors (2 mM CST, 100 μM KIF, 100 μM swainsonine and 10 nM concanamycin A), the cells were first incubated with these inhibitors for 1 h before the onset of radiolabelling and were then added to both the pulse and chase media at appropriate concentrations. BFA (10 μg/ml) was included only in the chase media. CHO and Lec23 cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 5 g/l glucose and 10% FCS. The cells were then pulse-radiolabelled with 50 μCi [2-^{3}H]mannose for 30 min in glucose-free Dulbecco's modified Eagle's medium supplemented as described above for glucose-free RPMI 1640. Chase incubations were performed as described above. In one of the experiments, CHO and Lec23 cells were labelled overnight with 500 μCi [2-^{3}H]mannose in complete growth medium.

Cell permeabilization

At the end of pulse–chase experiments, media from each incubation were stored frozen and the cells were washed with ice-cold PBS before being permeabilized with SLO as described previously [22]. Briefly, HepG2 cells were released from tissue culture flasks with trypsin/EDTA and washed twice in 1 ml of permeabilization buffer [PB; 250 mM mannitol/5 mM Hepes (pH 7.3)/2 mM EGTA/1 mM $CaCl_2$/2 mM $MgCl_2$]. Cells were incubated at 4 °C for 20 min in 0.5 ml PB containing 1 unit/ml SLO. Cells were recovered by centrifugation and the supernatant was retained. Subsequent to washing the cells twice with 0.5 ml PB at 4 °C, they were incubated with 0.5 ml pre-warmed PB (37 °C) for 5 min and the permeabilized cells were then recovered by centrifugation to yield the membrane-bound compartment fraction. The final supernatant was pooled with the SLO-containing supernatant and two subsequent PB washes to yield 2 ml of the cytosolic compartment fraction (cytosol).

Preparation and purification of FOS from cell fractions of HepG2 cells

Neutral FOS were prepared from the cell fractions as described previously [27,29]. Briefly, pellets of permeabilized or intact cells were extracted with a mixture of chloroform/methanol/125 mM Hepes (pH 7.2) containing 4 mM $MgCl_2$ (3:2:1, by vol.), and after vigorous shaking the upper methanolic phases were recovered, dried and redissolved in water. This material and the cytosol fractions were desalted on columns of AG 50-X2 (H^+ form) and AG 1-X2 (acetate form); unbound neutral material was then loaded on to charcoal columns, which were washed with water before elution of oligosaccharide material from the charcoal with 30% ethanol.

Preparation of FOS and glycoproteins from cell-culture media

Trichloroacetic acid (TCA; 1 ml, 25%, w/v) was added to the medium; subsequent to incubation for 1 h at 4 °C, the proteins were precipitated by centrifugation. The supernatant was recovered and washed four times with 4 ml diethyl ether before being dried and desalted on Bio-Gel P2. The protein pellets were treated with pronase (2 mg/ml) in 50 mM ammonium bicarbonate buffer (pH 8) for 24 h at 37 °C.

conA–Sepharose chromatography

The desalted TCA supernatant material and glycopeptides from the pronase digests were analysed by conA–Sepharose chromatography as described previously [26]. conA columns (1 ml) were equilibrated with 50 mM Tris/HCl (pH 7.8) buffer containing 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.02% (w/v) sodium azide. After allowing the sample to interact with the immobilized lectin for 30 min, elution was started with 10 ml of equilibration buffer (fraction I containing complex multi-

antennary glycans). Bound glycopeptides and FOS were eluted with 10 ml of 10 mM methyl-α-D-glucopyranoside (fraction II containing complex bi-antennary glycans), 10 mM methyl-α-D-mannopyranoside (fraction III containing hybrid-type glycans) and 500 mM methyl-α-D-mannopyranoside pre-warmed at 60 °C (fraction IV containing polymannose-type glycans) added sequentially in equilibration buffer. Fractions were desalted on a Bio-Gel P2 column.

Quaternary aminoethyl (QAE) fractionation of oligosaccharides

conA–Sepharose run-through fractions (fraction I) were loaded onto QAE–Sephadex columns (0.8 ml) in 2 mM Tris base, as described previously [30]. Subsequent to washing the columns with 7×750 μl of 2 mM Tris base, oligosaccharides were eluted in a batchwise manner with 20, 70, 125, 200, 400 and 1000 mM NaCl in 2 mM Tris base.

Separation of FOSGN1 and FOSGN2

FOSGN2 and FOSGN1 were separated as described previously [24] after derivatization with 2-aminopyridine [31]. After subsequent treatment of derivatized oligosaccharides with endo H and resolution of the digestion products on AG 50-X2 (H^+ form) columns, endo H-sensitive oligosaccharylaminopyridines were recovered as their FOSGN1 counterparts in the water effluent, whereas endo H-resistant oligosaccharylaminopyridines were eluted from the AG 50-X2 with 0.2 M NH_4OH.

TLC of OS

FOS larger than $Man_5GlcNAc$ were analysed on plastic TLC plates coated with silica, which were developed in *n*-propanol/acetic acid/water (3:3:2, by vol.) for 24 h, whereas smaller FOS were analysed on cellulose-coated plates that were developed in pyridine/ethyl acetate/water/acetic acid (5:5:3:1, by vol.) for 24 h. Resolved components were either visualized by fluorography or by spraying silica-coated plates with H_2SO_4/orcinol and heating at 100 °C for 10 min.

Enzyme reactions

C. perfringens sialidase digestions were performed by incubating oligosaccharide components in 100 mM acetate buffer (pH 5) containing 0.2 unit of enzyme for 15 h at 37 °C in a final vol. of 50 μl. Oligosaccharides were treated with 20 units alkaline phosphatase in 20 μl of 100 mM Tris/HCl (pH 8.0) for 15 h at 37 °C. The endo H digestions were performed by incubating glycopeptides, oligosaccharides or 2-aminopyridine oligosaccharides with 1.5 m-units of endo H in 20 μl sodium citrate buffer (pH 5.5) for 15 h at 37 °C.

RESULTS

FOS material is found in the medium of CST-treated HepG2 cells

As our previous *in vitro* studies have shown that triglucosylated FOSGN2 are unable to undergo ER-to-cytosol transport [15], the fate of triglucosylated FOSGN2 generated in the ER was examined. In particular, we wanted to know whether or not treatment of HepG2 cells with the glucosidase inhibitor CST could stimulate of FOSGN2 along the secretory pathway. In order to achieve this, cells were pulse-radiolabelled with [2-^{3}H]-mannose in the absence or presence of CST and/or the Golgi α-mannosidase inhibitor KIF, then the cells were chased in the absence or presence of these inhibitors for 8 h. The chase media were recovered and deproteinated by the addition of TCA. After centrifugation, the TCA-soluble material was subjected to Bio-Gel P2 chromatography, and the radioactive material eluting in the void volume (of molecular mass $\geqslant 2$ kDa) was collected and freeze-dried. This material, along with the pronase digests of the TCA-precipitated proteins, was subjected to conA–Sepharose chromatography as shown in Figure 1. Inspection of the left-hand side panels (precipitate) indicates that under control conditions HepG2 cells secrete glycoproteins bearing predominantly complex multi-antennary *N*-glycans (fraction I), and that in the presence of CST there is only a slight inhibition of complex *N*-glycan formation (fraction I) leading to a modest increase in the amount of N-linked polymannose-type oligosaccharides (fraction IV). By contrast, when a pulse–chase experiment was performed in the presence of KIF alone, we observed a dramatic inhibition of the appearance of complex N-linked glycans concomitant with a large accumulation of oligomannose-type *N*-glycans (fraction IV). These results are in good agreement with previous studies [27], which have shown that in HepG2 cells Golgi endo-α-D-mannosidase provides a glucosidase-independent pathway for the deglucosylation of glycoproteins, thereby allowing complex glycoprotein formation to proceed in the

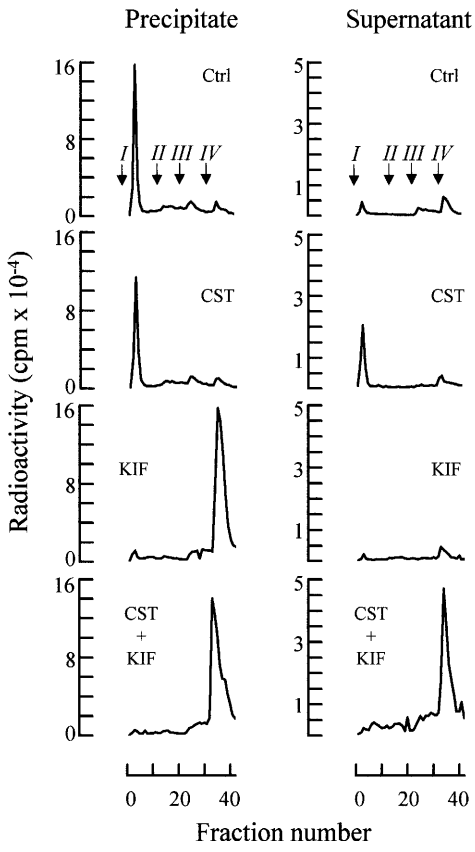

Figure 1 conA–Sepharose chromatographic analysis of TCA-soluble material and pronase digests of TCA-insoluble material from the chase medium of control, CST, KIF and CST + KIF-treated HepG2 cells

Control (ctrl), CST-treated and CST + KIF-treated HepG2 cells were pulse-radiolabelled with [2-^{3}H]mannose and chased in the continued presence of the glycosidase inhibitors for 8 h. The TCA-soluble material as well as the pronase digests of the TCA-precipitated proteins from the chase media were subjected to conA–Sepharose chromatography. The column run-through fractions (fraction I) are known to contain complex-type multiantennary glycans. Complex-type biantennary glycans were eluted with 10 mM methyl-α-D-glucopyranoside (fraction II), whereas hybrid-type glycans were eluted with 10 mM methyl-α-D-mannopyranoside (fraction III). Finally, polymannose-type glycans were eluted with 500 mM methyl-α-D-mannopyranoside pre-warmed to 60 °C (fraction IV).

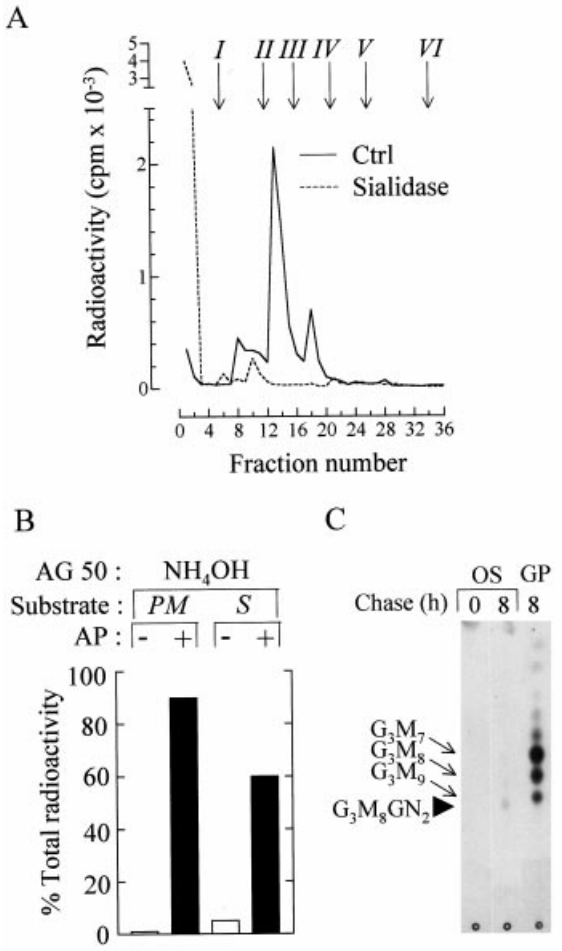

Figure 2 Analysis of TCA-soluble radioactivity recovered from the medium of CST-treated cells

(**A**) The TCA-soluble conA–Sepharose run-through material obtained from the medium of CST-treated cells (see Figure 1) was subjected to QAE–Sephadex ion-exchange chromatography before and after sialidase treatment as described in the Materials and methods section. Oligosaccharides were eluted with increasing concentrations of NaCl (fraction I, 20 mM; fraction II, 70 mM; fraction III, 125 mM; fraction IV, 200 mM; fraction V, 400 mM; and fraction VI, 1000 mM). (**B**) A standard mixture of FOS ($Man_{9-8}GlcNAc_2$, PM) and the radioactive material neutralized by sialidase treatment (S), as shown above, were subjected to reductive amination with 2-aminopyridine, and the reaction products were applied to AG 50 columns. After washing the columns with water, positively charged material was eluted with 200 mM NH_4OH. The water washes and NH_4OH eluates were quantified by scintillation counting, and the quantity of positively charged material is expressed as a percentage of the total. (**C**) TLC resolution of TCA-soluble (OS) and endo H-treated TCA-insoluble (GP), conA-positive material (fraction IV; see Figure 1) obtained from the medium of CST-treated HepG2 cells. Whereas the entire FOS fraction was loaded on to the TLC plate, only 44% of the GP material was examined. Abbreviations: $G_3M_8GN_2$, $Glc_3Man_8GlcNAc_2$; G_3M_9, $Glc_3Man_9GlcNAc$; G_3M_8, $Glc_3Man_8GlcNAc$; G_3M_7, $Glc_3Man_7GlcNAc$.

presence of the glucosidase inhibitor CST. When the TCA-soluble material (supernatant) was analysed in a similar manner, we noted that the presence of CST stimulated a 7-fold increase in complex multi-antennary oligosaccharide-like material (fraction I) compared with that observed in the medium derived from control cells. It is evident that the radioactive material failing to bind to the lectin was indeed generated by the N-linked glycosylation pathway; as seen in Figure 1 (bottom right-hand panel, CST + KIF), the mannosidase inhibitor blocked the CST-induced generation of the conA–Sepharose run-through material and, at the same time, it stimulated the appearance of substantial amounts of oligomannose-type oligosaccharide-like material (fraction IV). Importantly, KIF alone did not increase the amount of TCA-soluble material detected in the medium when compared with that found in the absence of inhibitors (control). Altogether, these results indicate that CST stimulates an appearance of TCA-soluble oligosacharide-like material in the medium of HepG2 cells.

Characterization of TCA-soluble oligosaccharide-like material recovered from the medium of CST-treated cells

CST-induced TCA-soluble conA–Sepharose run-through (Figure 1, fraction I) material was desalted on Bio-Gel P2. Preliminary experiments revealed that although it did not bind to a cation-exchange resin, $> 90\%$ bound to an anion exchanger, indicating that it was negatively charged (results not shown). The nature of this negative charge was further investigated by QAE–Sephadex ion-exchange chromatography before and after sialidase treatment. Results shown in Figure 2(A) indicate that most of the oligosaccharide material can be eluted with 70 mM NaCl, indicating that it has two negative charges [30]. Sialidase treatment causes 80% of the negatively charged oligosaccharide material to become neutral, demonstrating that sialic acid residues confer the negative charge on the oligosaccharide-like material. To demonstrate the oligosaccharide nature of this material, we attempted to show that it possessed a free reducing terminus; this was achieved by subjecting the sialidase-treated material to reductive amination with 2-aminopyridine. As shown in Figure 2(B), this procedure introduces a positive charge into standard $Man_{9-8}GlcNAc_2$ FOS with 90% efficiency, whereas pyridylamination of the sialidase-treated conA–Sepharose run-through oligosaccharide-like material recovered from the medium of CST-treated cells was 60% successful. Therefore, taking into account the efficiency of the reductive amination towards known FOS, it is apparent that at least 70% of the latter material comprises FOS. Figure 1 demonstrates that in addition to conA–Sepharose run-through TCA-soluble material being present in the medium obtained from CST-treated cells, small amounts of radioactivity were also recovered from the fractions known to contain structures bearing polymannose-type oligosaccharide units. After desalting, this fraction was found to contain an FOS that co-migrated with $Glc_3Man_8GlcNAc_2$. Endo H digestions of the polymannose-type glycopeptides generated from the TCA-precipitated medium proteins recovered from the same chase medium yielded several oligosaccharide species, with the major component co-migrating with standard Glc_3Man_7-GlcNAc during TLC. Finally, due to the small quantities of radioactivity available for analysis, we did not examine the medium from control incubations for sialic acid-containing FOS. However, despite the detection of radioactivity corresponding to TCA-soluble polymannose-type FOS material during conA–Sepharose chromatography, after TLC analysis we were not able to detect polymannose-type FOS in the medium of control or KIF-treated HepG2 cells cultivated in the absence of CST (results not shown). The small amounts of radioactive material observed to occur in fractions I and IV after lectin chromatography of the TCA-soluble radioactive material recovered from the medium of control and KIF-treated cells may correspond to small glycoproteins or glycopeptides that are not precipitated by TCA. To summarize, CST stimulates the appearance of both complex sialic acid-containing FOS and triglucosylated polymannose-type FOS in the medium of HepG2 cells.

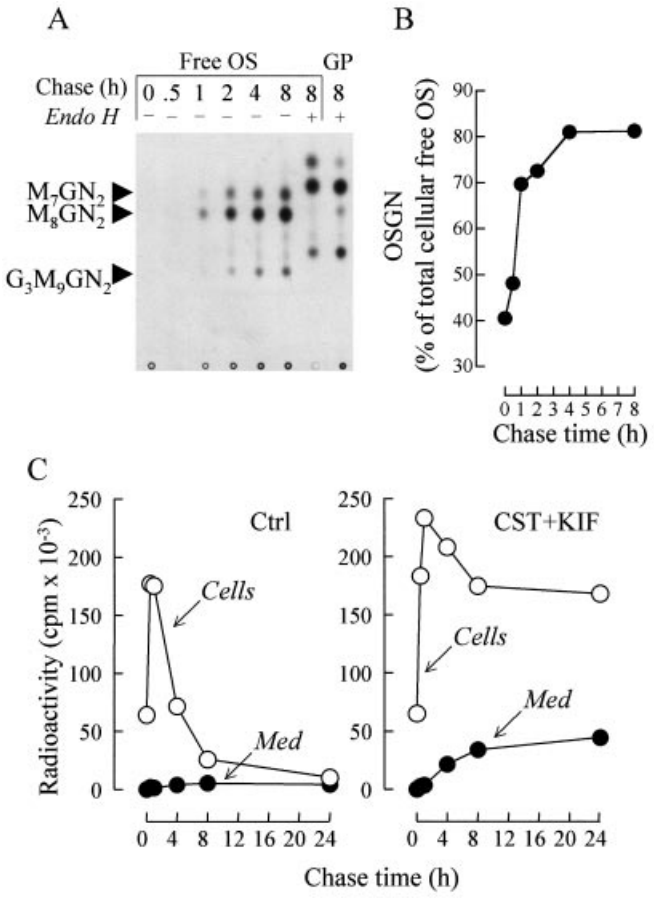

Figure 3 Analysis of TCA-soluble radioactivity recovered from the medium of CST + KIF-treated cells

CST + KIF-treated HepG2 cells were pulse-radiolabelled with [2-^{3}H]mannose and chased for different periods in the presence or absence of the two glycosidase inhibitors. (**A**) TLC of FOS isolated from media obtained at different chase times. At the 8 h chase time, the TCA-insoluble material (GP) was digested with pronase before endo H treatment, and 2% of the released oligosaccharides were analysed by TLC. (**B**) FOS were recovered from the cells at the different chase periods and derivatized with 2-aminopyridine before endo H treatment in order to determine the quantity of FOS possessing a single GlcNAc at the reducing terminus (FOSGN1), as detailed in the Materials and methods section. (**C**) In a separate experiment performed as described above, FOS were recovered from the cells and media (Med) of incubations conducted in the absence (control) or presence of the glycosidase inhibitors (CST + KIF). Abbreviations: $G_3M_9GN_2$, $Glc_3Man_9GlcNAc_2$; M_8GN_2, $Man_8GlcNAc_2$; M_7GN_2, $Man_7GlcNAc_2$.

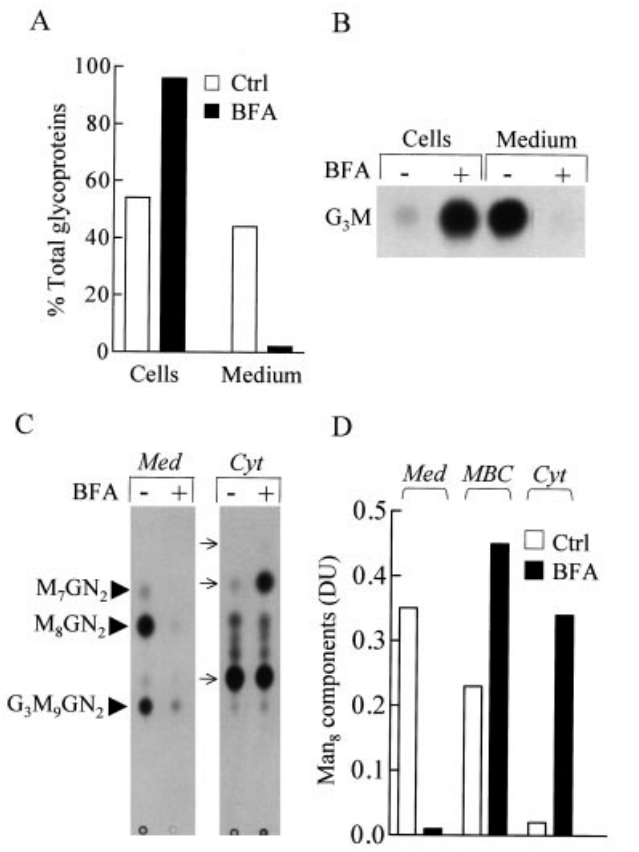

Figure 4 Effects of BFA on the subcellular trafficking of FOS in CST + KIF-treated HepG2 cells

CST + KIF-treated HepG2 cells were pulse-radiolabelled with [2-^{3}H]mannose and chased for 8 h in the presence of BFA. (**A**) Glycoproteins were recovered from the medium and cells, and after pronase digestion they were quantified by scintillation counting. (**B**) The tetrasaccharide Glc_3Man (G_3M) generated by Golgi endomannosidase was recovered from the cells and medium, before resolution from other oligosaccharide components by TLC on cellulose-coated plates. (**C**) In a separate experiment in which the CST + KIF-treated cells were pulse-radiolabelled and chased for 4 h, FOS were recovered from the media (Med), the cytosolic compartments (Cyt) and membrane-bound compartments (MBC) after SLO permeabilization of cellular plasma membranes. TLC of FOS recovered from the media and cytosolic compartments of control and BFA-treated cells are shown. (**D**) From the same experiment, $Man_8GlcNAc$ and $Man_8GlcNAc_2$ components were quantified by scanning densitometry. The abbreviations used are as described in the legend of Figure 3.

Characterization of TCA-soluble material recovered from the medium of CST + KIF-treated cells

Figure 1 shows that when cells are treated with CST and the mannosidase inhibitor KIF, the appearance of complex sialic acid containing FOS is blocked, and at the same time there is an appearance of oligosaccharide-like material bearing polymannose-type oligosaccharide structures. These TCA-soluble oligomannose-containing components (see Figure 1) were found to be neutral after ion-exchange chromatography and behaved as FOS after resolution by TLC. There is a time-dependent appearance of these FOS in the medium of CST + KIF-treated cells, and when these components were treated with endo H, the resulting species were observed to co-migrate with standard $Glc_3Man_9GlcNAc$, $Man_8GlcNAc$ and $Man_7GlcNAc$ oligosaccharides (Figure 3A). Although FOS recovered from the medium of CST + KIF-treated cells were uniquely FOSGN2, the intracellular FOS recovered from the same incubations revealed that the ratio of FOSGN1 to FOSGN2 changed throughout the chase period with the former species comprising 80% of the total cellular FOS after 4 h of chase (Figure 3B). The exclusive appearance of FOSGN2 in the medium of CST + KIF-treated cells indicates that these species reach the extracellular space by a mechanism that does not involve the cytoplasm or lysosomes, as these two subcellular compartments are known to possess endo H and chitobiase activities [20,21,32]. Furthermore, the preponderance of $Man_{8-7}GlcNAc_2$ species in the medium indicates that most of the FOSGN2 recovered from the medium of CST + KIF-treated cells are deglucosylated by endomannosidase, attesting to their transit through the Golgi apparatus. Finally, Figure 3(C) shows that during a pulse–chase experiment performed in the presence of CST + KIF, 20% of the total cellular FOS is secreted. In contrast, in uninhibited HepG2 cells FOS are metabolized inside the cell, and are not detected in the extra-

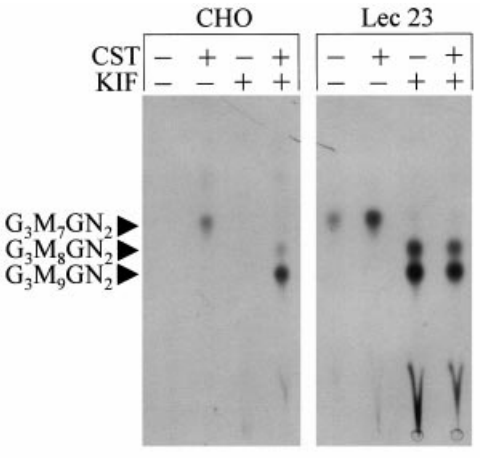

Figure 5 TLC of FOS obtained from the medium of CHO and Lec23 cells

CHO (left panel) and Lec23 (right panel) cells were pulse-radiolabelled with [2-^{3}H]mannose and chased for 8 h in the presence of the indicated glycosidase inhibitors. FOS were recovered from the chase media and subjected to TLC. The abbreviations used are as described in the legends of Figures 2 and 3.

cellular space (Figures 1 and 3C). To summarize, the ensemble of these results suggests that when FOS cannot be cleared from the ER into the cytosol, as expected under normal conditions, these components can enter ER-to-Golgi transport vesicles and gain access to the secretory pathway.

BFA blocks FOSGN2 secretion and stimulates the appearance of $Man_8GlcNAc$ in the cytosol of CST + KIF-treated HepG2 cells

To test our model further for FOS transport in mammalian cells, we have examined the effect of BFA, a membrane flux perturbant that blocks Golgi-mediated protein secretion, on FOSGN2 in glucosidase-inhibited cells. BFA blocks the classical secretory pathway and causes the fusion of various elements of the Golgi apparatus with the ER [33,34]. We reasoned that this agent should inhibit the appearance of FOSGN2 in the extracellular medium of CST + KIF-treated cells, and additionally, the deglucosylating action of Golgi endomannosidase, now relocated to the ER, could favour FOSGN2 deglucosylation, thereby enabling their eventual transport into the cytosol. To perform this experiment, pulse–chase experiments were carried out in the presence of 100 μM swainsonine, in addition to CST + KIF, so as to block demannosylation of any FOS appearing in the cytosol [22]. In addition, the vacuolar ATPase inhibitor conA was also included in these incubations in order to block the generation of FOS in the lysosomal compartment [22]. As demonstrated in Figures 4(A) and 4(B), BFA inhibited the secretion of both glycoproteins and the tetrasaccharide Glc_3Man, characteristic of Golgi endomannosidase action, by $> 90\,\%$ when compared with the secretion of these components observed in the absence of this drug. Furthermore, BFA stimulated the appearance of substantial amounts of $Man_8GlcNAc$ in the cytosolic compartment without affecting the amounts of cytosolic $Glc_3Man_9GlcNAc$. In fact, summation of the amounts of $Man_8GlcNAc$ and $Man_8GlcNAc_2$ species observed to occur in the medium, cytosol and membrane-bound compartments of BFA-treated cells indicated that this drug caused a 1.5-fold increase in Man_8 components when compared with that found to occur in cells treated with the glycosidase inhibitors alone. Furthermore, of the total amount of Man_8 components observed in the presence of BFA, $40\,\%$ were found to occur in the cytosol. Our interpretation of these results is that in the presence of BFA endomannosidase-mediated deglucosylation of glucosylated FOSGN2 in the ER enables the resulting $Man_{8-7}GlcNAc_2$ oligosaccharides to be cleared from this organelle into the cytosol where they are acted upon by the cytosolic chitobiase to yield $Man_{8-7}GlcNAc$ species.

FOS are secreted from glucosidase I-deficient cells

Next, we examined whether or not the secretion of FOSGN2 was a unique property of CST-treated HepG2 cells. Accordingly, the fate of FOS in CST-treated CHO cells and the glucosidase I-deficient CHO cell, Lec23 [26], was examined. Figure 5 (left panel) demonstrates that FOSGN2 are recovered from the medium of CST-treated CHO cells, but not their untreated or KIF-treated counterparts. Because CHO cells possess little detectable endomannosidase activity these cells secrete predominantly triglucosylated FOSGN2. In contrast, as shown in Figure 5 (right panel), FOSGN2 (predominantly $Glc_3Man_7GlcNAc_2$) were recovered from the medium of Lec23 cells grown in the absence of CST. We noted that incubation of CST-treated CHO cells and untreated Lec23 cells with KIF increased the amount of FOSGN2 recovered from the chase media, indicating that, probably, these cell lines are capable of synthesizing (triglucosylated) sialylated hybrid-type FOSGN2 structures, as has been demonstrated for the biosynthesis of glycoproteins in these two cell lines [26]. In summary, it is apparent that the capacity of triglucosylated FOSGN2 to follow the secretory pathway during ER glucosidase deficiency is not restricted to the HepG2 cell line.

Intracellular FOS accumulation in glucosidase I-deficient cells

Finally, we examined the extent of intracellular FOS accumulation in the Lec23 cell line. Although we show that FOSGN2 generated in the lumen of the ER can be secreted from glucosidase I-compromised cells it is known that triglucosylated FOSGN1 can accumulate in the cytosolic compartment of CST-treated HepG2 cells [24]. These cytosolic FOSGN1 are now thought to be generated in the cytosol by the action of deglycosylating enzymes on misfolded glycoproteins that have been translocated out of the ER into the cytosol via the sec61-containing protein-conducting channel situated in the ER membrane [17,18]. In contrast with ER-to-cytosol transport of FOSGN2, the translocation of misfolded glycopeptides out of the ER into the cytosol is unaffected by the glucosylation status of the peptide-borne N-linked oligosaccharides [35,36]. To evaluate the accumulation of FOS generation in glucosidase I-deficient cells, CHO and Lec23 cells were radiolabelled for 16 h in complete growth medium. Figure 6(A) shows that under normal cell-growth conditions the Lec23 cell line accumulates substantial quantities of FOS as revealed by both fluorography after metabolic radiolabelling, and by orcinol/H_2SO_4 visualization of FOS harvested from non-radiolabelled cells. The most abundant FOS was found to co-migrate with $Glc_3Man_5GlcNAc$, the species known to accumulate in the cytosol of CST-treated HepG2 cells [24]. In contrast, under the same conditions we were not able to detect the presence of triglucosylated FOS in the parental CHO line. However, both the parent and mutant cell lines revealed the presence of FOS species that co-migrated with $Man_5GlcNAc$ and $Man_6GlcNAc/Glc_1Man_5GlcNAc$. Next, the pile up of FOS in Lec23 cells as a function of cell growth was examined. Cells were harvested 3, 6 and 8 days after seeding into tissue culture flasks, and FOS were prepared from the cells and

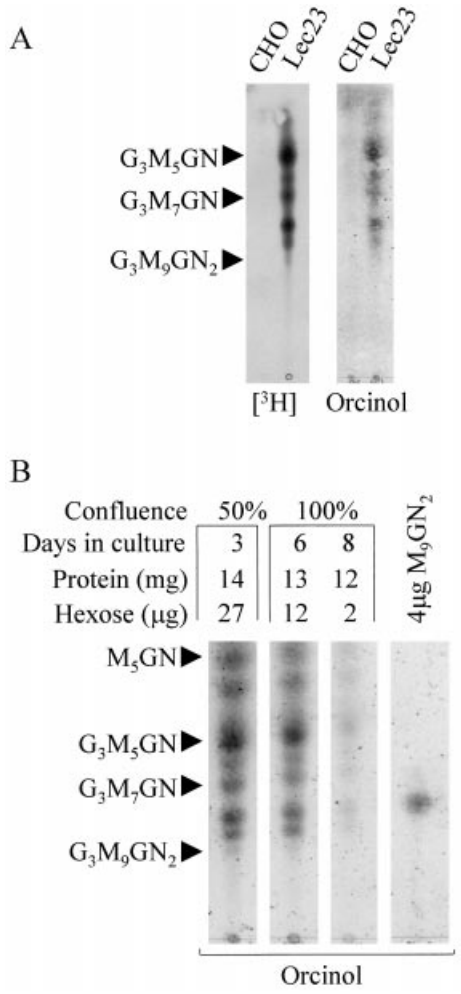

Figure 6 TLC examination of FOS in CHO and Lec23 cells

(**A**) Cells were metabolically radiolabelled with [2-^{3}H]mannose for 24 h as described in the Materials and methods section, and the cellular FOS were extracted and analysed by TLC [^{3}H] Three confluent 75 cm^2 tissue culture flasks of each cell line were harvested. After desalting and separation from monosaccharides on charcoal, the extracted cellular FOS were resolved by TLC and visualized by the orcinol/H_2SO_4 reagent (Orcinol). (**B**) Lec23 cells were seeded into tissue culture flasks, and harvested at the indicated times (days in culture). At each time point, cells (4×75 and 2×75 cm^2 flasks for 50 and 100% confluent cells respectively) were extracted and FOS were analysed by TLC as described above. The amount of total hexose applied to each lane is indicated, as well as the amount of total cellular protein recovered from the tissue culture flasks. The abbreviations used are as defined above.

quantified by the orcinol/H_2SO_4 assay. FOS derived from approx. 13 mg cellular protein equivalents were then analysed by TLC as shown in Figure 6(B). It is apparent that FOS accumulation is maximal during cell growth and decreases slowly after cells reach confluence.

DISCUSSION

In the present study, we examined the role of triglucosylated FOS that are generated in glucosidase I-compromised cells. We provide three pieces of evidence demonstrating that ER-generated triglucosylated FOS, whose clearance into the cytosol is blocked, follow the secretory pathway before appearing in the extracellular space. First, extracellular FOS were found to comprise uniquely FOSGN2 species, indicating that they escape from the cell without passing through the cytosol or lysosomal compartments, both of which are known to contain chitobiase activities. Secondly, the bulk of extracellular FOSGN2 were found to have structures resembling the ones resulting from a passage through the Golgi apparatus. Thirdly, BFA inhibited the appearance of extracellular FOSGN2 in glucosidase-inhibited HepG2 cells and also caused an intracellular accumulation of these structures. Indeed, our experiments with BFA strongly suggest that the ability of this drug to fuse elements of the Golgi apparatus with the ER favours endomannosidase-mediated deglucosylation of ER-situated triglucosylated FOSGN2 thereby stimulating the appearance of the deglucosylated $Man_{7-8}GlcNAc$ species in the cytosol (Figure 4C). At present, our results do not allow us to say whether or not FOSGN2 are transported along the secretory pathway by bulk flow [37], or by a process mediated by lectins thought to be involved in the ER-to-Golgi transport of certain glycoproteins [38–40]. Although, as described above, most of our observations are consistent with the secretion of FOS by the classical secretory pathway, other data indicated that our interpretation of the results may be too simplistic. For example, we observed that Golgi processing of FOSGN2 was not complete, because in the presence of both CST and CST + KIF, we noted that not all extracellular FOSGN2 had been deglucosylated by Golgi endomannosidase (see Figures 2C and 3A). This result was surprising as it is generally thought that incomplete Golgi processing of N-linked glycans is due to intramolecular glycan/polypeptide interactions that hinder the approach by glycosidases or glycosyltransferases. In addition, we also noted that, whereas the inhibitory effect of BFA on the secretion of glycoproteins Glc_3Man and $Man_{7-8}GlcNAc_2$ was $> 90\%$, the inhibitory effect of this reagent on the secretion of free $Glc_3Man_{9-8}GlcNAc_2$ species was consistently less pronounced (see Figure 4C). This fact coupled with the previous observation, suggesting that some FOS might escape Golgi processing, may indicate that FOSGN2 can follow an alternative Golgi-independent secretory pathway linking the ER to the cell surface. With this observation in mind it is interesting to note that experiments conducted on the secretion of glycosylated tripeptides generated in hepatocytes of perfused mouse liver revealed BFA to be ineffective on the rate of glycopeptide appearance in the liver perfusate. However, the glycotripeptides recovered from the BFA perfusate were found to be endo H-sensitive, indicating that they may not have passed through the Golgi apparatus [41]. These authors proposed that perhaps, in the presence of BFA, small glycopeptides could take alternative routes to the cell surface such as that reported to occur for the BFA-insensitive ER-to-plasma membrane transport of cholesterol [42].

We have shown that FOSGN2 secretion occurs in both Lec23 cells and the CST-treated parental CHO cell line as well as in CST-treated HepG2 cells, indicating that this phenomenon might reasonably be thought to occur in all ER glucosidase I-compromised cells synthesizing N-type glycoproteins. In view of this proposal, it is surprising that FOSGN2 did not occur in the urine of the patient diagnosed with a deficiency in ER glucosidase I. In fact, examination of the reported data does not allow one to say with certainty whether or not complex-type FOSGN2 were present in the urine of this individual, but one possible explanation for the absence of these components is that complex, sialic acid-containing FOSGN2 may be efficiently removed from the bloodstream in the liver. Under normal circumstances, FOS are metabolized within the cell so that usually glycoproteins and glycolipids are the only extracellular carriers of oligosaccharide signals. As our experiments demonstrate that, in the absence of functional ER glucosidase I, FOSGN2 gain access to the secretory

pathway in intact cells, it is possible that these unconjugated structures could interfere with aspects of glycoprotein function in the extracellular space.

Next, we investigated the extent of intracellular FOS accumulation in glucosidase I-deficient cells in order to evaluate whether or not this phenomenon could have had detrimental effects on intracellular homoeostasis in the patient diagnosed with ER glucosidase I deficiency. Our results show that in CST-treated HepG2 cells only 20 % of the total cellular FOS can be secreted into the extracellular space, and that after 4 h of chase the bulk of intracellular FOS are FOSGN1, indicating that they are localized to intracellular compartments possessing either a chitobiase, or endo H-like activity. In fact, previous results [24] have shown that there is a pile up of triglucosylated FOSGN1 in the cytoplasm of CST-treated HepG2 cells. In the present study, we report that the triglucosylated FOS that accumulate inside Lec23 cells can be detected by orcinol/H_2SO_4 and results indicate that these structures could be recovered from the cells in quantities up to 60 nmol/g wet weight of cells. Calculating the amount of triglucosylated FOSGN2 in Lec23 cells (after jack-bean mannosidase digestion before and after endo H treatment of FOS; results not shown) indicated that up to 20 % of the total FOS in these cells may be present in the ER. Assuming that this organelle may occupy approx. 10 % of the cell volume, we calculate that the ER may contain up to 120 μM triglucosylated FOSGN2. Mouse liver may contain up to 4 nmol FOS/g wet weight [43], whereas ox thyroid has been estimated to contain up to 12 nmol FOS/g wet tissue [11]. By contrast, human skin biopsy fibroblasts obtained from a patient with lysososmal α-mannosidosis have been demonstrated to contain several tens of μmoles of FOS/g protein [44]. Accordingly, the cytosolic accumulation of triglucosylated FOSGN1 observed in Lec23 cells is unlikely to perturb cellular homoeostasis unless FOS play a still unrecognized and specific signalling role in cell metabolism. Furthermore, it is known that triglucosylated oligosaccharides N-linked to glycoproteins interact poorly, if at all, with many of the lectins situated along the secretory pathway [5,6,38,40]. Accordingly, the ER-situated, lectin-based chaperone system is largely inoperable in glucosidase I-deficient cells. Nevertheless, studies in which $Glc_1Man_9GlcNAc_2$ binding to calreticulin and calnexin have been examined indicate IC_{50} values of 1–5 μM for the tetrasaccharide Glc_1Man_3 [45]. Thus, under circumstances other than glucosidase I deficiency, in which ER-to-cytosol transport of FOSGN2 is blocked, the resulting accumulation of FOS in the ER could potentially interfere with lectin-based folding reactions in this organelle. Next, we studied whether or not this modest pile up of intracellular FOS increased with the time of cell culture. Surprisingly, we noted that the concentration of intracellular FOS declined in the Lec23 cell line after the cells reached confluence. At present it is not clear how triglucosylated FOS are eventually cleared from the Lec23 cell line although this process could be accomplished by the non-specific bulk delivery of cytosol to lysosomes by macroautophagy known to occur in these cells [9]. One intriguing possibility is that changes in cytosolic FOS concentrations, such as those reported here, occur in the Lec23 cell line and could themselves be involved in the regulation of mechanisms involved in either the generation (unfolded protein response, ER-associated protein degradation, protein glycosylation) or degradation (glycosidase expression, FOS transport) of these structures in mammalian cells [46]. Despite our observation that FOS accumulation in the Lec23 cell line was growth dependent, it is possible that much more dramatic FOS accumulations would be observed in cells, such as hepatocytes, which synthesize large quantities of glycoprotein in a growth-independent manner. Interestingly, the patient diagnosed with a deficiency in glucosidase I displayed a rapidly progressive hepatomegaly [25].

In summary, we showed that FOS trafficking is severely perturbed in cells where glucosidase I activity is compromised. For the first time we demonstrate that FOSGN2 generated in the lumen of the ER can follow the secretory pathway if their clearance from the ER into the cytosol is impeded. In addition, the results demonstrate that glucosidase I-deficient CHO cells accumulate 60 nmol cytosol-generated triglucosylated FOSGN1/g cells and that this pile up declines after the cells reach confluence. We are at present examining the eventual fate of cytosolic FOS, the lysosomal import of which is blocked, and investigating the possible role of cytosolic FOS in mammalian cell glycoprotein metabolism.

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