Cytosolic deglycosylation process of newly synthesized glycoproteins generates oligomannosides possessing one GlcNAc residue at the reducing end

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Recent studies on the mechanism of degradation of newly synthesized glycoproteins suggest the involvement of a retrotranslocation of the glycoprotein from the lumen of the rough endoplasmic reticulum into the cytosol, where a deglycosylation process takes place. In the studies reported here, we used a glycosylation mutant of Chinese hamster ovary cells that does not synthesize mannosylphosphoryldolichol and has an increased level of soluble oligomannosides originating from glycoprotein degradation. In the presence of anisomycin, an inhibitor of protein synthesis, we observed an accumulation of glucosylated oligosaccharide-lipid donors ($Glc_3Man_5GlcNAc_2$-PP-Dol), which are the precursors of the soluble neutral oligosaccharide material. Inhibition of rough endoplasmic reticulum glucosidase(s) by castanospermine led to the formation of $Glc_3Man_5GlcNAc_2$(OSGn2) (in which OSGn2 is an oligomannoside possessing two GlcNAc residues at its reducing end), which was then retained in the lumen of intracellular vesicles. Thus they were protected during an 8 h chase period from the action of cytosolic chitobiase, which is responsible for the conversion of OSGn2 to oligomannosides possessing one GlcNAc residue at the reducing end (OSGn1). In contrast, when protein synthesis was maintained in the presence of castanospermine, glucosylated oligomannosides ($Glc_{1-3}Man_5GlcNAc_1$) were recovered in cytosol. Except for monoglucosylated Man_5 species, which are potential substrates for luminal calnexin and calreticulin, the pattern of oligomannosides was similar to that observed on glycoproteins. The occurrence in the cytosol of glucosylated species with one GlcNAc residue at the reducing end implies that the deglycosylation process that generates glucosylated OSGn1 from glycoproteins occurs in the cytosol.

INTRODUCTION

The N-glycosylation process is accomplished through the co-translational transfer of a preassembled oligosaccharide ($Glc_3Man_9GlcNAc_2$) from a lipid carrier, dolichol, to a nascent polypeptide chain. It is now well established that the biosynthesis of N-glycosylproteins is accompanied by the release of free oligosaccharides. In various biological models, this material is composed mainly of oligomannosides possessing one or two GlcNAc residue(s) at the reducing end (OSGn1 and OSGn2) that are rapidly cleared from the rough endoplasmic reticulum (ER) lumen and degraded. Three subcellular compartments are involved in this catabolic pathway, i.e. rough ER, cytosol and lysosomes. Glucosylated OSGn2 originating from lipid intermediate cleavage are first deglucosylated in the rough ER and transported via a specific carrier into the cytosol [1,2], where the action of a cytosolic chitobiase [3] produces OSGn1 species, which are potential substrates for cytosolic α-D-mannosidase [4] leading to a single $Man_5GlcNAc$ isomer: Manα1-2Manα1-2Manα1-3(Manα1-6)Manβ1-4GlcNAc. This oligomannoside enters into the lysosome [5] to be further degraded into smaller species and monosaccharides.

Even though the main steps of this oligomannoside trafficking are known, the origin of these oligosaccharides is still questionable. By using radiolabelling of oligomannosides and with different biological models, two origins have been suggested: they originate either from the hydrolysis of oligosaccharide-PP-Dol [6] or from the degradation of newly synthesized glycoproteins [7,8].

The degradation of newly synthesized proteins and glycoproteins has been recently documented (reviewed in [9,10]). Recent studies have shown that misfolded glycoproteins are retrotranslocated from the rough ER lumen into the cytosol, then degraded by the proteasome after ubiquitination. This mechanism has been demonstrated for membrane glycoproteins [11–14] and soluble glycoproteins [15]. It has to be noted that this degradation process is the result of the action of a viral protein [11,12], of a quality control mechanism induced by a defect in the amino acid sequence [13,15] or by impaired association of subunits [14]. As a prerequisite of this degradation process, a deglycosylated intermediate has been observed in some models [11,12,14] and the action of a cytosolic peptide N-glycanase (PNGase) releasing OSGn2 has been suggested.

The B3F7 cell line is a glycosylation mutant of Chinese hamster ovary (CHO) cells that does not synthesize mannosylphosphoryldolichol, producing only truncated $Glc_{0-3}Man_5GlcNAc_2$-PP-Dol [16]. At a low glucose concentration (0.5 mM) and when cultured at 40 °C, the level of soluble oligomannosides released is higher in B3F7 cells than in wild-type CHO cells. This has been correlated with an increased glycoprotein degradation [8]. By using this model we demonstrate in the present paper that a cytosolic deglycosylation process of oligomannoside-type glycoproteins produces OSGn1.

MATERIALS AND METHODS

Reagents

Mutant cell line B3F7 was isolated from B 4-2-1, Lec15.1, which does not synthesize mannosylphosphoryldolichol [16]. [2-^{3}H]Mannose (429 Gbq/mmol) was from Amersham (Little

Abbreviations used: CHO, Chinese hamster ovary; ER, endoplasmic reticulum; OSGn1, oligomannoside possessing one GlcNAc residue at its reducing end; OSGn2, oligomannoside possessing two GlcNAc residues at its reducing end; PNGase, peptide N-glycanase.

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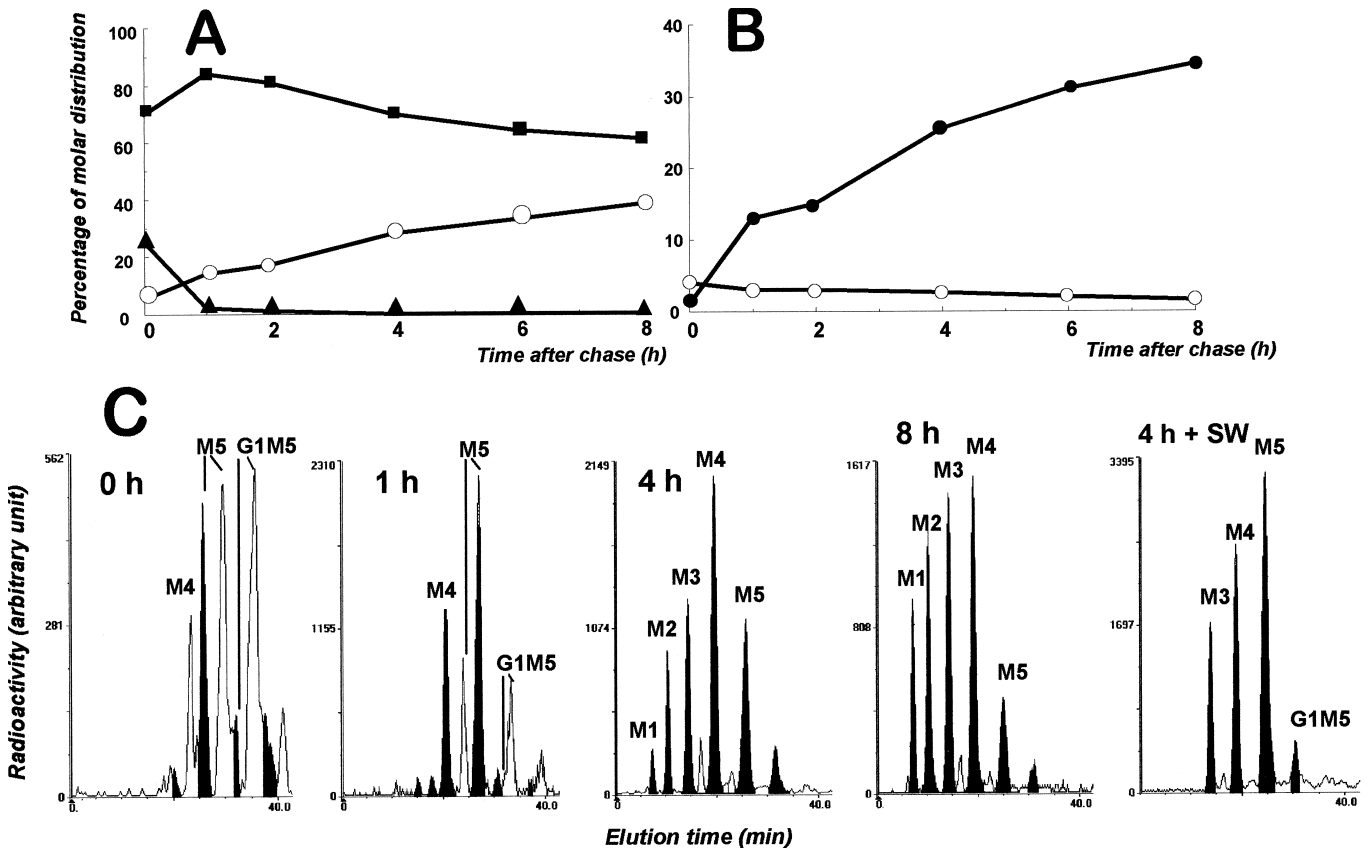


Figure 1 Oligomannoside trafficking in B3F7

B3F7 cells were pulsed with [2-^{3}H]mannose in the presence of 0.5 mM Glc for 30 min and chased for 1, 2, 4, 6 and 8 h at 40 °C. (**A**) Percentage molar distribution of the various fractions: oligosaccharide-lipids (▲), proteins (■) and soluble neutral oligomannosides (○). The molar equivalents of the fractions were calculated after HPLC analysis, taking into account the specific radioactivity of each peak. (**B**) Kinetics of the liberation of the free neutral oligosaccharide species expressed as a percentage of the molar distribution: ○, OSGn2; ●, OSGn1. (**C**) HPLC profiles of free neutral oligosaccharides after 0, 1, 4 and 8 h of chase respectively; 4 h + SW represents an HPLC profile of free oligosaccharides after a 4 h chase in the presence of 0.1 mM swainsonine. M1, M2, M3, M4, M5 and G1M5 indicate oligosaccharide species possessing one (filled peaks) or two (open peaks) GlcNAc residues at the reducing end, and one, two, three, four or five Man residues or one Glc and five Man residues respectively.

Chalfont, Bucks., U.K.). PNGase F, castanospermine and swainsonine were purchased from Boehringer Mannheim (Mannheim, Germany). Anisomycin and trypsin were obtained from Sigma (St. Louis, MO, U.S.A.). Concanavalin A–Sepharose was from Pharmacia.

Metabolic labelling of oligosaccharides, and chase experiments

B3F7 cells were routinely cultured in monolayers in α-minimal essential medium with 10 % (v/v) fetal calf serum at 34 °C in 10 cm Petri dishes under air/CO_2 (19:1).

For labelling of bound and free oligomannosides, B3F7 cells, cultured in 10 cm Petri dishes, were preincubated overnight at 40 °C. Cells were labelled at 40 °C with 100 μCi of [2-^{3}H]mannose per dish in α-minimal essential medium without glucose and 10 % (v/v) dialysed fetal calf serum. After incubation, the medium was removed and the cell layer was washed rapidly three times with ice-cold PBS. When a chase was performed, pulse-labelled cells were washed twice with PBS and incubated for different durations in α-minimal essential medium containing 5 mM glucose. When used, castanospermine was maintained throughout the incubation and the chase periods at a final concentration of 50 μg/ml. Sequential extraction was then achieved as described previously [1].

The radioactivity bound to oligosaccharide-PP-Dol and to glycoproteins was measured by liquid-scintillation counting. The free oligosaccharide material was purified as described [8].

Analysis of oligosaccharide material

The protein pellet was digested overnight at room temperature with 0.2 mg of trypsin treated with 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one in 0.1 M ammonium bicarbonate, pH 7.9. The glycan moiety linked to protein was cleaved by PNGase F as follows: the trypsin-treated protein was boiled for 10 min to destroy the trypsin activity and the peptides were dried and dissolved in 20 mM sodium phosphate, pH 7.5, containing 50 mM EDTA, 50 % (v/v) glycerol and 0.02 % NaN_3. PNGase F was then added (0.5 unit, corresponding to 0.5 mIU) for incubation overnight at 37 °C.

Size analysis of the glycan moieties and of the oligosaccharide material was achieved by HPLC on an amino-derivatized Asahipak NH2P-50 (250 mm × 4.6 mm) column (Asahi, Kawasaki-ku, Japan) with a solvent system of acetonitrile/water from 70:30 (v/v) to 50:50 (v/v) at a flow rate of 1 ml/min over 80 min. Under these conditions the oligomannosides can be resolved by their numbers of mannose, glucose and GlcNAc residues from $Man_1GlcNAc$ to $Glc_3Man_5GlcNAc_2$. Oligo-

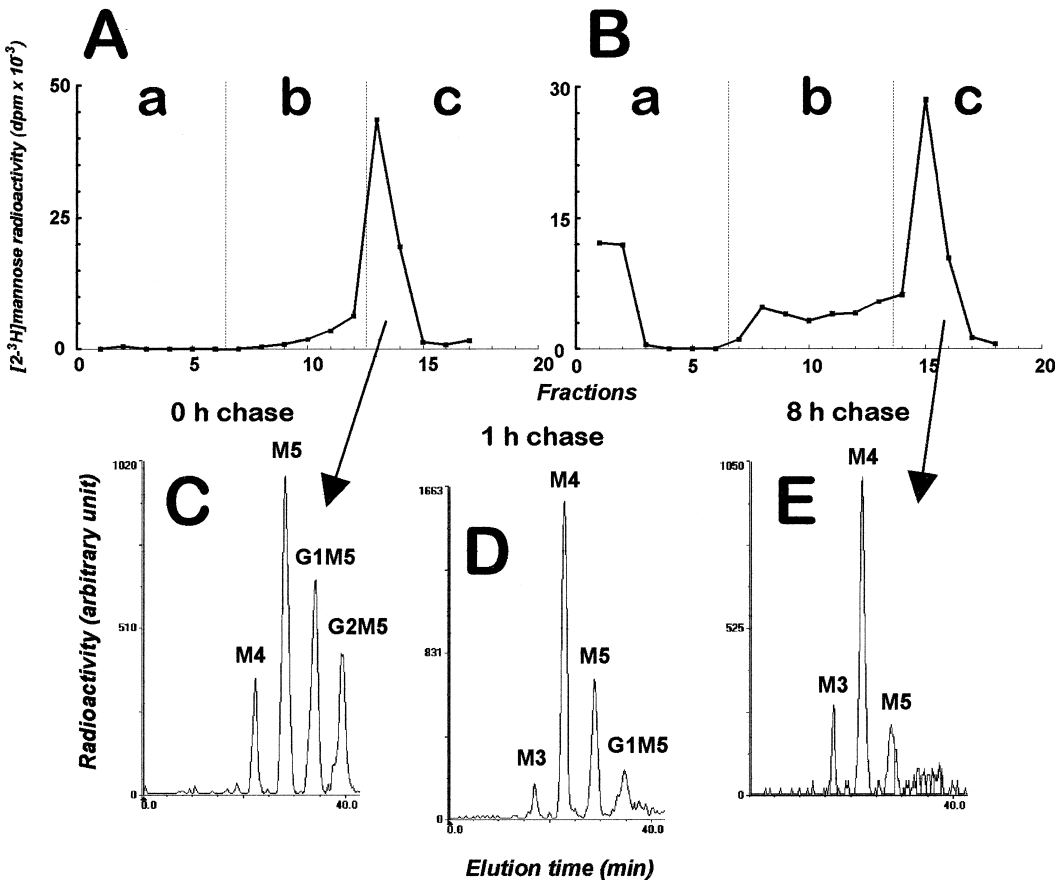


Figure 2 Concanavalin A–Sepharose chromatography and HPLC analysis of glycoproteins during a pulse–chase experiment

B3F7 cells were pulsed with [2-^{3}H]mannose in the presence of 0.5 mM Glc for 30 min and chased for 8 h at 40 °C. After sequential extraction the glycoprotein fraction was treated with PNGase and then subjected to concanavalin A–Sepharose chromatography. Regions labelled a, b and c represent the equilibration buffer alone (a), with 10 mM methyl α-D-glucoside (b) or 100 mM α-D-mannoside. (**A**, **B**) Affinity chromatography of glycan fractions after 0 h (pulse, **A**) and 8 h chase (**B**). (**C**–**E**) HPLC analysis of the fraction eluted in buffer c after 0 h (**C**), 1 h (**D**) and 8 h (**E**) of chasing. Peaks are identified as for Figure 1.

mannosides were identified as described previously [1] by their retention times; separation of the labelled oligosaccharides was monitored by continuous-flow detection of the radioactivity with a Flo-one β detector (Packard).

Cell permeabilization

B3F7 cells were routinely permeabilized by the method of Beckers et al. [17]. In brief, adherent cells were washed three times with an ice-cold swelling buffer made of 10 mM Hepes, pH 7.2, and 15 mM KCl. After incubation for 10 min on ice, the swelling buffer was replaced with 3 ml of breaking buffer [50 mM Hepes (pH 7.2)/90 mM KCl]. Cells were immediately scraped from the plate and resuspended in an isotonic buffer [30 mM Tris/HCl (pH 7.5)/120 mM KCl/4 mM magnesium acetate]. We have shown previously that this permeabilization technique does not affect intracellular membranes [1].

Affinity chromatography of labelled N-glycans

The lectin column (concanavalin A–Sepharose, 5 cm × 0.5 cm) was equilibrated in 5 mM sodium acetate buffer, pH 5.2, containing 0.1 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ at room temperature. The glycan fraction resulting from PNGase F action on the glycoprotein fraction was applied to the column, which was then eluted with the equilibration buffer (buffer a). The weakly retained glycans were eluted with 10 mM methyl α-D-glucoside in the equilibration buffer (buffer b); strongly retained glycans were eluted with 100 mM α-D-mannoside (buffer c).

RESULTS

Formation and trafficking of oligomannosides in B3F7 cells

The liberation of oligomannosides originating from the deglycosylation of newly synthesized glycoprotein was shown during a pulse–chase experiment with B3F7 cells. Figure 1 shows an 8 h chase experiment performed after a 30 min pulse with [2-^{3}H]mannose in the presence of 0.5 mM glucose. Figure 1(A) shows the percentage molar distribution of the various fractions after HPLC analysis. The molar equivalent of the resolved components was calculated by dividing the radioactivity of each peak by the number of mannosyl residues in the oligomannoside species; the molar equivalents were then summed and the percentage molar distribution was calculated. The oligosaccharide-PP-Dol fraction was chased rapidly (1 h) but it is clear that the molar equivalent of the soluble oligosaccharides continued to increase after 1 h, reaching 40% of the total incorporation after an 8 h chase. This liberation of an increasing

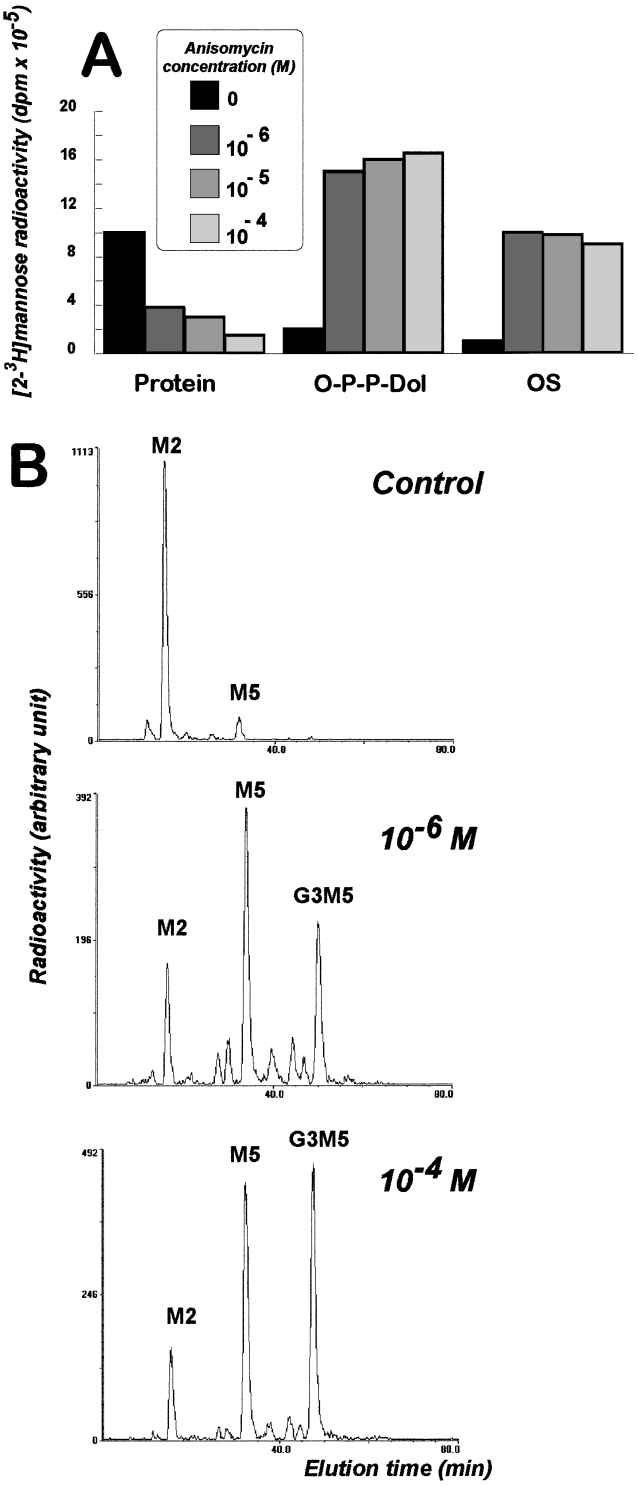


Figure 3 Effect of anisomycin on lipid-linked oligosaccharide synthesis in B3F7 cells

B3F7 cells were incubated with [2-^{3}H]mannose for 30 min under the conditions described in the Materials and methods section in an incubation medium without glucose, in the presence of various concentrations of anisomycin: 0, 1, 10 and 100 μM. (**A**) Effect of anisomycin on the incorporation into protein, oligosaccharide-PP-Dol (O-P-P-Dol) and soluble neutral oligomannosides (OS). (**B**) HPLC analysis of the oligosaccharide fraction released from oligosaccharide-lipid for the control and for 1 μM and 100 μM anisomycin. Peaks are identified as for Figure 1.

quantity of soluble oligosaccharides could not have originated uniquely from oligosaccharide-PP-Dol cleavage and was concomitant with the deglycosylation of the glycoprotein fraction. The analysis of the soluble oligosaccharide fractions in terms of OSGn1 or OSGn2 underlines the difference in the time progress curves for these two species (Figures 1B and 1C). From the beginning of the chase to 1 h, the OSGn2 species (mainly $Glc_{0-1}Man_5GlcNAc_2$) were released from the lipid intermediates pool and further converted into OSGn1 as shown at 1 and 4 h of chasing. The smaller species ($Man_2GlcNAc_1$ and $Man_1GlcNAc_1$) that appeared at 4 h corresponded to lysosomal degradation products, as demonstrated by Saint-Pol et al. [5] and by the fact that their formation was inhibited when the chase was performed in the presence of 0.1 mM swainsonine (see Figure 1C; 4 h + SW). From 4 to 8 h of chasing the only possible precursor for the formation of OSGn1 was the pool of glycoproteins. As is shown in Figure 1(B), a striking fact is that OSGn2, produced by oligosaccharide-PP-Dol hydrolysis during the first hour of chase, could not be the sole precursor of the large amount of OSGn1 liberated between 4 and 8 h.

In B3F7 cells the glycoprotein deglycosylation process seems to be rather slow compared with the high degradation rate described in some other models (a half-time of 1 or 2 min has been described for MHC class I heavy chains [11]). Figure 2 illustrates the type of glycan bound to glycoproteins by using affinity chromatography on a concanavalin A–Sepharose column. When analysed after a 30 min pulse, 90 % of the glycans were tightly retained by the lectin (Figure 2A) and the HPLC analysis revealed non-glucosylated ($Man_{4-5}GlcNAc_2$) and glucosylated ($Glc_{2-1}Man_5GlcNAc_2$) species (Figure 2C). After a 1 h chase, processing occurred leading to deglucosylation and partial demannosylation, because the main species was $Man_4GlcNAc_2$ (Figure 2D). After an 8 h chase, 48 % of the glycans were still retained by concanavalin A (Figure 2B), and the pattern of oligomannosides was similar to that observed after a 1 h chase (Figure 2E). This result demonstrated that sufficient amounts of oligomannoside-type glycoproteins are retained in B3F7 to generate OSGn1, for at least 8 h, by a deglycosylation process.

Thus both the oligosaccharide-PP-Dol and glycoprotein deglycosylation processes generate oligomannosides in B3F7. To discriminate between these two origins we studied oligomannoside formation in the presence of anisomycin, a protein synthesis inhibitor.

Effect of anisomycin on the pattern and fate of the lipid-linked oligosaccharide precursors

Figure 3(A) shows the effect of various concentrations of anisomycin, an inhibitor of the ribosomal translocation reaction, on the synthesis, composition and fate of oligosaccharide-PP-Dol. As expected, when B3F7 cells were incubated in the absence of glucose with various concentrations (from 1 to 100 μM) of this protein synthesis inhibitor, the transfer of the radiolabelled oligosaccharide-PP-Dol to protein was decreased, leading to an 8-fold accumulation of the precursor. Part of this excess of labelled lipid intermediates was used by the oligosaccharyltransferase to generate neutral oligosaccharides, because a 10-fold increase in the radioactivity bound to the soluble neutral oligosaccharide material was observed in the presence of anisomycin (Figure 3A). The analysis of glycans bound to lipid intermediates is shown in Figure 3B. The main species recovered in the control were $Man_2GlcNAc_2$ (80 %) and $Man_5GlcNAc_2$ (7 %), as described previously [18]. As already observed with puromycin or cycloheximide [19], anisomycin-treated cells accumulated

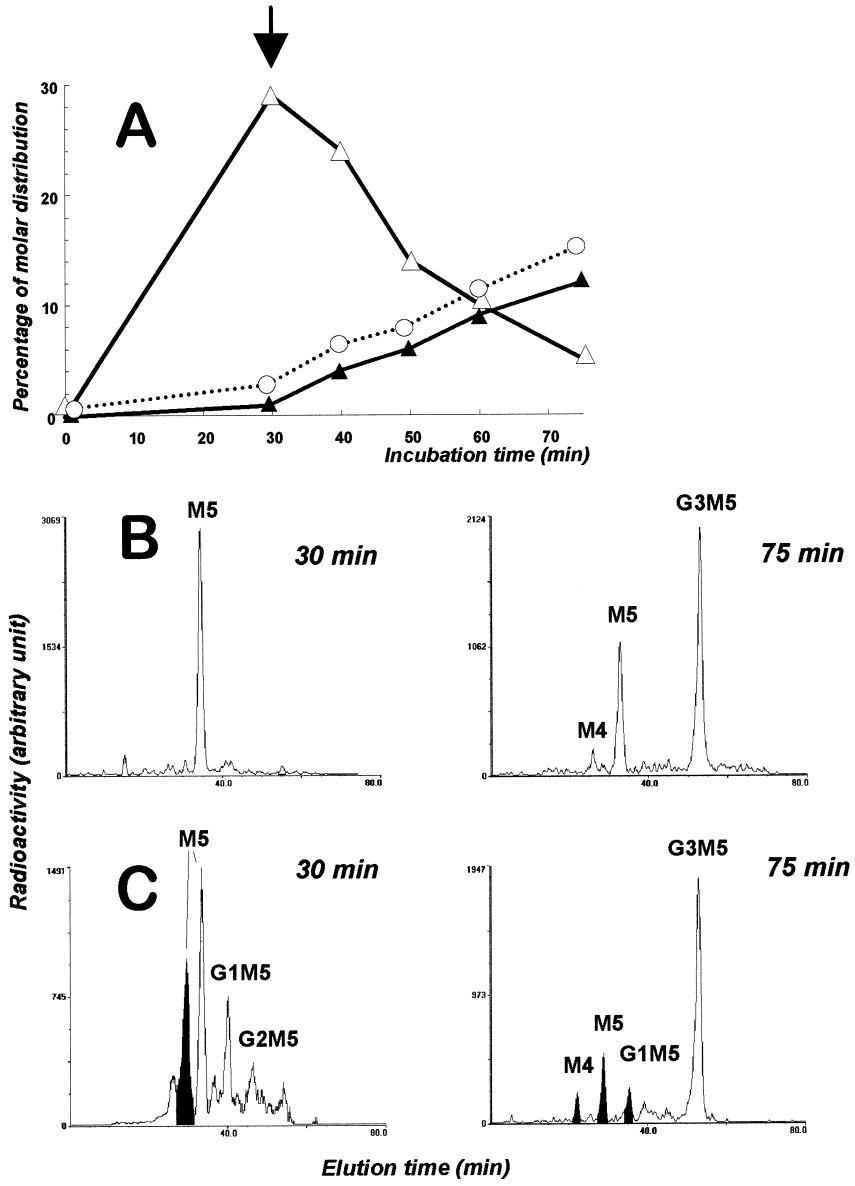


Figure 4 Fate of glucosylated oligomannosides in B3F7

(**A**) B3F7 cells were pulsed with [2-^{3}H]mannose under the conditions described in the Materials and methods section. After 30 min, 100 μM anisomycin and 50 μg/ml castanospermine (arrow) were added for a further 10, 20, 30 and 45 min of incubation. After the incubation, cells were subjected to sequential extraction and the oligosaccharide-lipids were submitted to mild acid hydrolysis and analysed by HPLC. (**A**) Percentage molar distributions of $Man_5GlcNAc_2$-PP-Dol (△), $Glc_3Man_5GlcNAC_2$-PP-Dol (▲) and neutral soluble oligomannosides (○). (**B**) HPLC analysis at 30 and 75 min of the oligosaccharide moieties linked to oligosaccharide-PP-Dol. (**C**) HPLC analysis of the neutral oligomannoside material after 30 and 75 min of incubation. Peaks are identified as for Figure 1.

predominantly a glucosylated species, $Glc_3Man_5GlcNAc_2$, reaching 45% of the radioactivity at 100 μM anisomycin. This species is the metabolic end product of the lipid intermediate synthesis in this Man-P-Dol-deficient cell line. It demonstrated that anisomycin-treated cells exhibit an accumulation of glucosylated species. These glucosylated species, which are more efficiently transferred to protein acceptor [20], are not used under these conditions of protein synthesis inhibition.

In the absence of protein synthesis, $Glc_3Man_5GlcNAc_2$-PP-Dol is the only precursor of soluble neutral oligosaccharide material

B3F7 cells were metabolically labelled with radioactive mannose in the presence of 0.5 mM glucose at 40 °C. After 30 min of incubation, 100 μM anisomycin and 50 μg/ml castanospermine (an inhibitor of rough ER glucosidases) were added. The incubation then continued for 0–45 min. Figures 4(A) and 4(B) show the analysis of glycans bound to oligosaccharide-lipids before and after the addition of anisomycin. As observed in Figure 4(B), after the first 30 min of incubation $Man_5GlcNAc_2$-PP-Dol was the only species detected. The addition of anisomycin produced very rapidly (half-time 20 min) the accumulation of fully glucosylated end product: $Glc_3Man_5GlcNAc_2$-PP-Dol at the expense of the non-glucosylated intermediate species $Man_5GlcNAc_2$-PP-Dol. After 45 min in the presence of anisomycin, only 12% (expressed as a molar distribution) of $Glc_3Man_5GlcNAc_2$-PP-Dol was detected at the expense of 24% of $Man_5GlcNAc_2$-PP-Dol. The difference was entirely recovered

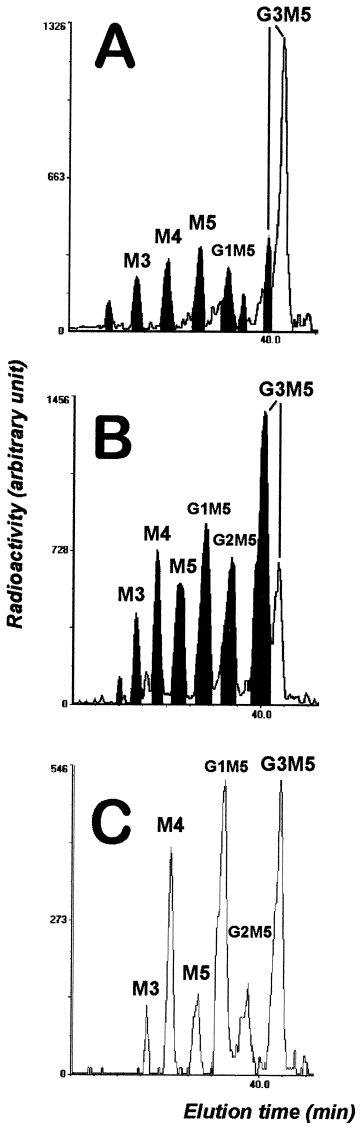


Figure 5 Pattern of soluble oligomannosides originating from the deglycosylation of glycoproteins in the presence of castanospermine

B3F7 cells were pulsed with [2-^{3}H]mannose under the conditions described in the Materials and methods section in the presence of 50 μg/ml castanospermine, with (**A**) or without (**B**, **C**) 100 μM anisomycin. After a 4 h chase the neutral oligosaccharide fraction was analysed by HPLC. (**A**) HPLC analysis of the neutral oligomannoside material synthesized in the presence of anisomycin and castanospermine. (**B**) HPLC analysis of the neutral oligomannoside material synthesized in the absence of anisomycin. (**C**) HPLC analysis after a 4 h chase of the glycan moieties bound to proteins synthesized in the presence of castanospermine. M3, M4, M5, G1M5, G2M5 and G3M5 indicate oligosaccharide species possessing one (filled peaks) or two (open peaks) GlcNAc residues at the reducing end, and three, four or five Man residues or one, two or three Glc plus five Man residues respectively.

as soluble $Glc_3Man_5GlcNAc_2$. As demonstrated in Figure 4(C), $Glc_3Man_5GlcNAc_2$ represented 80 % of the radioactivity in the soluble oligomannoside fraction, the remaining 20 % having been released during the first 30 min of incubation. Thus the experiment with anisomycin and castanospermine was in agreement with the fact that the fate of glucosylated oligosaccharide-PP-Dol was hydrolysis to glucosylated OSGn2. Owing to the inhibition of glucosidases by castanospermine, these glucosylated oligomannosides could not be deglucosylated and therefore could

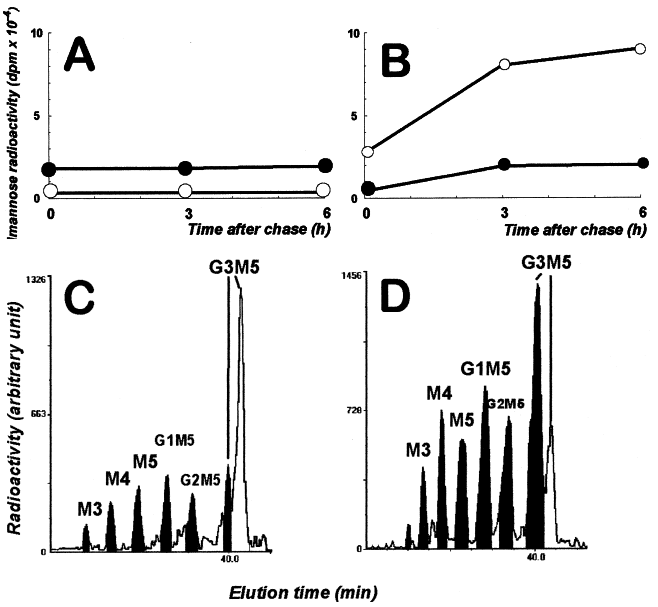


Figure 6 Subcellular location of OSGn1 and OSGn2 synthesized in the presence of castanospermine

B3F7 cells were pulsed with [2-^{3}H]mannose in the presence of 0.5 mM Glc and 50 μg/ml castanospermine for 30 min and chased for 0, 3 or 6 h at 40 °C. After the chase, cells were permeabilized as described in the Materials and methods section and washed by low-speed centrifugation. The free oligomannoside material was purified from the supernatant (○). The cell pellet was subjected to sequential lipid extraction and the soluble cell-associated oligosaccharides were recovered from the aqueous phase (●). In these two compartments the kinetics of the liberation of OSGn2 (**A**) and OSGn1 (**B**) was determined. (**C**, **D**) HPLC analysis, at 6 h of chase, of the cell-associated (**C**) and cytosolic (supernatant) (**D**) neutral free oligomannosides. Peaks are identified as for Figure 1.

not be transported into the cytosol to be precursors of OSGn1, as has been observed in HepG2 cells [2].

Pattern of oligomannosides originating from glycoprotein deglycosylation in the presence of castanospermine

Figure 5 shows the pattern of soluble oligosaccharide material released after a 4 h chase period in the presence of castanospermine and in the presence (Figure 5A) or absence (Figure 5B) of anisomycin. When protein synthesis was inhibited (5 % of the incorporated radioactivity was bound to glycoproteins) 80 % of the released oligomannoside species was $Glc_3Man_5GlcNAc_2$ as demonstrated above and illustrated in Figure 5(A). In contrast, when protein synthesis was maintained (61.5 % of the radioactivity was incorporated into glycoproteins during the 30 min pulse), the soluble oligomannosides were mainly composed of OSGn1 species (80 %), of which 60 % were glucosylated (Figure 5B). The pattern was similar to that recovered on glycoproteins (Figure 5C). Because the chitobiase activity responsible for the conversion of OSGn2 into OSGn1 is located in the cytosol [3], and because glucosylated species are not transported into the cytosol, the occurrence of glucosylated OSGn1 when protein synthesis is allowed seems questionable. Thus we decided to study the subcellular location of the oligosaccharides released during glycoprotein synthesis in the presence of castanospermine.

Subcellular location of the released oligomannosides

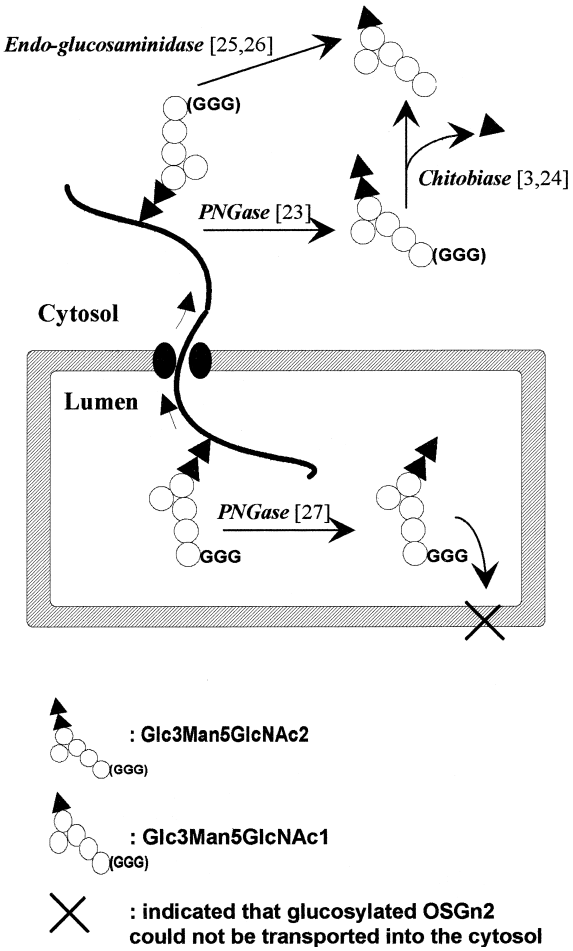
Figure 6 shows the results of a pulse–chase experiment achieved with [2-^{3}H]mannose in the presence of castanospermine. After a

30 min pulse the cells were chased for 0, 3 or 6 h. Before sequential extraction, cells were permeabilized and the oligomannosides were recovered either from the supernatant (cytosolic location) or from the cell pellet (vesicular compartment), as demonstrated previously [1]. OSGn2 species were recovered predominantly from the vesicular compartment (75%) (Figure 6A). The level was constant during the chase, demonstrating that they could not exit from rough ER because they consisted mainly of $Glc_3Man_5GlcNAc_2$ (Figure 6C) originating from oligosaccharide-PP-Dol hydrolysis. In contrast, OSGn1 were mainly recovered from the cytosol (85%), the total amount increasing during the chase. The pattern of OSGn1 observed in the cytosol (Figure 6D) was similar to that obtained by analysing glycans of glycoproteins under the same conditions (Figure 5C). This result is in complete agreement with the fact that deglycosylation of newly synthesized glycoprotein occurs in the cytosol, releasing oligomannosides terminating in a single GlcNAc residue.

DISCUSSION

During the N-glycosylation process, the mature glucosylated oligosaccharide-PP-Dol is utilized by oligosaccharyltransferase for transfer to acceptor proteins or for generating luminal OSGn2. In addition it has been shown that OSGn2 could be converted to OSGn1 via a cytosolic chitobiase, which implies the transfer of OSGn2 into the cytosol. B3F7 is a Man-P-Dol synthase-deficient CHO cell line and consequently these cells synthesize Man_5-type glycans. This glycosylation defect seems to induce the retention of some glycoproteins, as previously demonstrated for a recombinant human alkaline phosphatase (SeAP) when expressed in the CHO glycosylation mutant MadIA214. In this model, SeAP bearing truncated glycans ($Glc_1Man_5GlcNAc_2$) was maintained for at least 8 h in the cell and the glycoprotein co-precipitated with a marker for rough ER, the molecular chaperone BiP [21], indicating that impaired glycosylation could affect some folding steps in the rough ER. This retention could supply the glycoprotein substrates for a slow deglycosylation process as a prerequisite of the degradation of misfolded proteins, as demonstrated for some models [11,12,14].

The nature and location of oligomannoside species originating from the deglycosylation process has been studied by using anisomycin and castanospermine. These inhibitors led to the accumulation of the fully glucosylated oligosaccharide-PP-Dol, which was not used for protein glycosylation. The oligomannoside part of the oligosaccharide lipid was released (presumably by the hydrolytic activity of the oligosaccharyltransferase [6]) and was accumulated in the lumen of intracellular vesicles as $Glc_3Man_5GlcNAc_2$ owing to the inhibition of the glucosidases by castanospermine and because the chitobiase activity responsible for the cleavage of the chitobiosyl-unit of OSGn2 has been demonstrated to be cytosolic [3]. After a 4 h chase in the presence of both inhibitors, only a small percentage (10%) of $Glc_3Man_5GlcNAc_1$ was detected; this demonstrated that, as previously described, glucosylated oligomannosides cannot be transported out of the rough ER [2]. When the chase experiment was performed without anisomycin, 60% of the radioactivity bound to soluble oligomannosides was from the OSGn1 species, even when these species were glucosylated. In addition the same oligomannoside species were found in glycoproteins and in OSGn1, suggesting a substrate–product relationship, but there are some differences in their proportions. When the pattern of protein-linked glycans (Figure 5C) and the pattern of OSGn1 recovered in the cytosol (Figure 6D) are compared, the major difference is in the Glc_1Man_5 species. It can be proposed that glycoproteins bearing Glc_1Man_5 species are protected from deglycosylation. As recently demonstrated by Vassilakos et al. [22], the Glcα1-3Manα1-2Manα1-2Man structure extending from the α1,3 branch of the oligosaccharide core is sufficient for recognition by calnexin and calreticulin. This motif is present in glycans synthesized by B3F7. The association between glycoprotein and these molecular chaperones could allow their retention, thus avoiding their retrotranslocation and deglycosylation.



Scheme 1 Retrotranslocation of glycoproteins and location of the various oligosaccharide-releasing enzymes

The scheme has been adapted for the B3F7 model from the results of Wiertz et al. [11,12]. References corresponding to the demonstration of the subcellular location and isolation of enzymes are shown.

The subcellular location of the released oligomannosides has been studied by using the permeabilization method. Analysis of the free neutral oligomannosides from the vesicular and from the cytosolic compartment shows that $Glc_3Man_5GlcNAc_2$ was again the main species remaining within the intracellular vesicles throughout the chase; in contrast, $Glc_{1-3}Man_5GlcNAc_1$ were the main species detected in the cytosol and their quantities increased during the chase. This indicates that OSGn1 originated from glycoprotein deglycosylation but were not produced in the lumen of rough ER.

This raises questions about the nature of the enzyme involved in the deglycosylation process and its subcellular location. We observed the degradation of a rough ER/*cis*-Golgi-located glycoprotein releasing OSGn1 into the cytosol. According to

Wiertz et al. [11,12], deglycosylation occurs in the cytosol by the action of a PNGase releasing OSGn2 after translocation of glycoprotein in the cytosol. If a PNGase such as that described by Suzuki et al. [23] is involved, the intermediate OSGn2, which was not detectable in our model, should be immediately converted to OSGn1 by a chitobiase, which has been shown to be present in the cytosol [3] and isolated recently from hen oviduct [24]. Another possibility is the action of the cytosolic endoglucosaminidase demonstrated in the rat liver by Pierce et al. [25,26]. This latter hypothesis, which fits best with our observations, remains to be addressed by following the fate and trafficking of a given misfolded glycoprotein. A PNGase activity has recently been described in the lumen of rat liver microsomes [27]. This enzyme could liberate luminal OSGn2, precursors of OSGn1, after transportation into the cytosol. In our view the presence of glucosylated OSGn1 in the cytosol is not in accord with this hypothesis because glucosylated species are not translocated. A proposal for the retrotranslocation of glycoproteins and the location of the various oligosaccharide-releasing enzymes is given in Scheme 1.

Whatever enzymes were involved, the released glycans followed the catabolic pathway of free oligomannosides until they reached the lysosome for the final degradation steps.

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REFERENCES

1 Kmiécik, D., Herman, V., Stroop, C. J. M., Michalski, J. C., Mir, A. M., Labiau, O., Verbert, A. and Cacan, R. (1995) Glycobiology **5**, 483–494
2 Moore, S.E. H., Bauvy, C. and Codogno, P. (1995) EMBO J. **14**, 6034–6042
3 Cacan, R., Dengremont, C., Labiau, O., Kmiécik, D., Mir, A. M. and Verbert, A. (1996) Biochem. J. **313**, 597–602
4 Grard, T., Herman, V., Saint-Pol, A., Kmiécik, D., Labiau, O., Mir, A. M., Alonso, C., Verbert, A., Cacan, C. and Michalski, J. C. (1996) Biochem. J. **316**, 787–792
5 Saint-Pol, A., Bauvy, C., Codogno, P. and Moore, S. E. H. (1997) J. Cell. Biol. **136**, 45–49
6 Spiro, M. J. and Spiro, R. G. (1991) J. Biol. Chem. **266**, 5311–5317
7 Anumula, K. R. and Spiro, R. G. (1983) J. Biol. Chem. **258**, 15274–15282
8 Villers, C., Cacan, R., Mir, A. M., Labiau, O. and Verbert, A. (1994) Biochem. J. **298**, 135–142
9 Bonifacino, J. S. and Lippincott-Schwartz, J. (1991) Curr. Opin. Cell. Biol. **3**, 592–600
10 Bonifacino, J. S. (1996) Nature (London) **384**, 405–406
11 Wiertz, E. J. H. J., Jones, T. R., Sun, L., Bogyo, M., Geuze, H. J. and Ploegh, H. L. (1996) Cell **84**, 769–779
12 Wiertz, E. J. H. J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T. R., Rapoport, T. A. and Ploegh, H. L. (1996) Nature (London) **384**, 432–438
13 Ward, C.L, Omura, S. and Kopito, R. R. (1995) Cell **83**, 121–127
14 Hughes, E. A., Hammond, C. and Cresswell, P. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 1896–1901
15 Qu, D., Teckman, J. H., Omura, S. and Perlmutter, D. H. (1996) J. Biol. Chem. **271**, 22791–22795
16 Stoll, J. (1986) PhD thesis, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore
17 Beckers, C. J. M., Keller, D. S. and Balch, W. E. (1987) Cell **50**, 523–534
18 Cacan, R., Villers, C., Bélard, M., Kaiden, A., Krag, S. S. and Verbert, A. (1992) Glycobiology **2**, 127–136
19 Chapman, A. E. and Calhoun, J. C. (1988) Arch. Biochem. Biophys. **260**, 320–333
20 Turco, S. J., Stetson, B. and Robbins, P. W. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 4411–4414
21 Ermonval, M., Cacan, R., Gorgas, K., Haas, I. G., Verbert, A. and Buttin, G. (1997) J. Cell. Science **110**, 323–336
22 Vassilakos, A, Michalak, M., Lehrman, M. A. and Williams, D. B. (1998) Biochemistry **37**, 3480–3490
23 Suzuki, T., Seko, A., Kitajima, K., Inoue, Y. and Inoue, S. (1994) J. Biol. Chem. **269**, 17611–17618
24 Kato, T., Hatanaka, K., Mega, T. and Hase, S. (1997) J. Biochem. **122**, 1167–1173
25 Pierce, R. J., Spik, G. and Montreuil, J. (1979) Biochem. J. **180**, 673–676
26 Pierce, R. J., Spik, G. and Montreuil, J. (1980) Biochem. J. **185**, 261–264
27 Weng, S. and Spiro, R. G. (1997) Biochem. J. **322**, 655–661

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