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Endoplasmic reticulum-to-cytosol transport of free polymannose oligosaccharides in permeabilized HepG2 cells

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Free polymannose oligosaccharides have recently been localized to both the vesicular and cytosolic compartments of HepG2 cells. Here we investigated the possibility that free oligosaccharides originating in the lumen of the endoplasmic reticulum (ER) are transported directly into the cytosol. Incubation of permeabilized cells in the absence of ATP at 37°C led to the intravesicular accumulation of free $Man_9GlcNAc_2$ which was generated from dolichol-linked oligosaccharide in the ER. This oligosaccharide remained stable within the permeabilized cells unless ATP was added to the incubations at which time the $Man_9GlcNAc_2$ was partially converted to $Man_8GlcNAc_2$, and both these components were released from an intravesicular compartment into the cytosolic compartment of permeabilized cells. In contrast, when permeabilized cells, primed with either free triglucosyl-oligosaccharide or a glycotripeptide, were incubated with ATP both these structures remained associated with the intravesicular compartment. As the conditions in which free oligosaccharides were transported out of the intravesicular compartment into the cytosolic compartment did not permit vesicular transport of glycoproteins from the ER to the Golgi apparatus our data demonstrate the presence of a transport process for the delivery of free polymannose oligosaccharides from the ER to the cytosol.

Keywords: cytosol/endoplasmic reticulum/Golgi apparatus/polymannose oligosaccharides/transport

Introduction

N-glycans play important roles in the folding and subcellular trafficking of glycoproteins (Helenius, 1994; Fiedler and Simons, 1995). Recently, calnexin, a transmembrane protein resident in the endoplasmic reticulum (ER) (Bergeron *et al.*, 1994), has been shown to be a chaperone involved in the folding of glycoproteins whose oligosaccharide chains bear single glucose residues (Hebert *et al.*, 1995). Another resident ER protein, calreticulin, also has lectin-like properties (Hebert *et al.*, 1995) and functions as a molecular chaperone during the biosynthesis of myeloperoxidase (Nauseef *et al.*, 1995). In addition, the mannose-binding lectin MR60 (Carpentier *et al.*, 1994) is now known to be identical to the ER–Golgi-intermediate compartment marker protein ERGIC-53 (Schindler *et al.*, 1993; Arar *et al.*, 1995). Although MR60 was first identified by its ability to bind bovine serum albumin–mannose conjugates (BSA–man) (Pimpaneau *et al.*, 1991) neither its oligosaccharide-binding specificity nor its function is known. Furthermore, MR60/ERGIC-53 shows substantial homology to VIP-36 (Fiedler and Simons, 1994), an integral membrane protein that is found in the Golgi apparatus and is thought to recycle between this compartment and the plasma membrane (Fiedler *et al.*, 1994). Accordingly, it has been speculated that the functions of MR60/ERGIC-53 and VIP-36 involve their interaction with glycoconjugates in transit through the secretory pathway (Fiedler and Simons, 1995).

As well as the synthesis of glycoconjugates, cells generate substantial quantities of free oligosaccharides in the lumen of the endoplasmic reticulum (Anumula and Spiro, 1983; Cacan *et al.*, 1987) which potentially could also interact with lectins along the secretory pathway. It is known that a proportion of these free polymannose oligosaccharides are released from dolichol-PP–oligosaccharide and terminate at their non-reducing end with a di-*N*-acetylchitobiose moiety (Spiro and Spiro, 1991; Villers *et al.*, 1994). Presently, neither the mechanism nor the reason for the release of polymannose oligosaccharides from dolichol into the lumen of the ER is understood.

Moreover, nothing is known about the subcellular trafficking of free polymannose oligosaccharides. In theory, these components could be substrates for the bulk flow of material leaving the ER for the Golgi apparatus. Indeed, the rate of bulk flow has been estimated by examining the rate of secretion of a polymannose-type glycotripeptide formed in the ER (Wieland *et al.*, 1987). However, initial observations concerning the fate of free oligosaccharides revealed that these components are not secreted into the tissue culture medium (Moore and Spiro, 1990). This surprising result raised the question as to why different fates are met by, on the one hand, a glycotripeptide formed in the lumen of the ER, and on the other hand, a free oligosaccharide formed in the same compartment. More recent studies have demonstrated the presence of substantial amounts of free polymannose-type oligosaccharides in the cytosolic compartment of permeabilized HepG2 cells (Moore and Spiro, 1994).

Whether or not free oligosaccharides perform a function within the lumen of the ER, their accumulation in this compartment may be deleterious to the proper functioning of the complex machinery (comprising lectins, glycosidases and glycosyltransferases) involved in the folding and trafficking of glycoproteins along the secretory pathway. Accordingly, the ER may have developed strategies for the clearance of free oligosaccharides from its interior.

In the present report, using permeabilized HepG2 cells

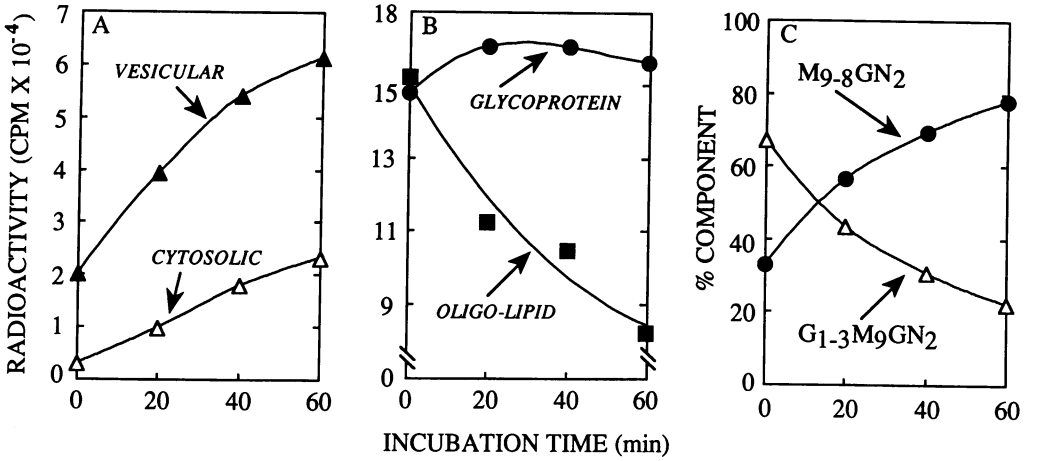

Fig. 1. Generation of free oligosaccharides in permeabilized HepG2 cells. PCs were incubated in transport buffer and an ATP-degenerating system for the indicated times at 37°C. (**A**) After termination of the incubations the PCs, representing the intravesicular compartment (*VESICULAR*), were separated from the incubation medium, representing the cytosolic compartment (*CYTOSOLIC*), and free oligosaccharides were isolated from each fraction as described in Materials and methods. (**B**) The PCs were further extracted to yield an oligosaccharide–lipid fraction (*OLIGO-LIPID*) and a delipidated protein pellet. The protein pellets were digested with pronase and aliquots were taken for scintillation counting (*GLYCOPROTEIN*). (**C**) Free oligosaccharides recovered from the vesicular compartment of PCs (*VESICULAR*, panel A) were resolved by thin-layer chromatography as described in Materials and methods. Oligosaccharides co-migrating with standard non-glucosylated, $Man_{8-9}GlcNAc_2$ ($M_{9-8}GN_2$), and glucosylated oligosaccharides $Glc_{1-3}Man_9GlcNAc_2$ ($G_{1-3}M_9GN_2$) were eluted from the thin-layer plate and quantitated by scintillation counting. The radioactivity associated with each class of component was summed at each incubation time and the percentage non-glucosylated and glucosylated components have been calculated.

incubated in the presence of ATP, we have demonstrated for the first time that soluble oligosaccharides are transported out of the lumen of the ER into the cytosolic compartment.

Results

Free polymannose oligosaccharides are generated from dolichol-PP–oligosaccharides in permeabilized HepG2 cells

HepG2 cells were pulse-radiolabelled for 20 min and then permeabilized with streptolysin O (SLO) as described in Materials and methods. When the permeabilized cells (PCs) are subsequently incubated for 60 min at 37°C in transport buffer as shown in Figure 1 there is a 3-fold increase in the quantity of soluble oligosaccharide material associated with the intravesicular compartment of the PCs and also an accumulation of free oligosaccharides in the cytosolic compartment (Figure 1A). The total increase in quantity of soluble oligosaccharide material could be accounted for by the decrease in radioactivity associated with the oligosaccharide–lipid (Figure 1B). At the same time, no change in the quantity of radioactivity associated with glycoproteins was observed (Figure 1B). Analysis of the reducing termini of the released free oligosaccharides generated by the PCs revealed that they exclusively occurred as the di-*N*-acetylchitobiose moiety (results not shown). Furthermore, thin-layer chromatography of the free oligosaccharides isolated from the PCs revealed that at the zero time point these components were mainly glucosylated ($Glc_{1-3}Man_9GlcNAc_2$) but after the 60 min incubation it is apparent that the non-glucosylated ($Man_{8-9}GlcNAc_2$) free oligosaccharides predominated within the vesicular compartment of the PCs (Figure 1C). At the same time we were unable to detect any demannosylation of the free oligosaccharides (results not shown). Figure 1 demonstrates that when radiolabelled PCs are incubated in the absence of ATP there is an accumulation of large free polymannose-type oligosaccharides in the vesicular compartment of the PCs. This observation is in contrast to previous *in vivo* observations (Moore and Spiro, 1994), where it was shown that free oligosaccharides terminating with the di-*N*-acetylchitobiose unit are extremely short-lived in both the vesicular and cytosolic compartments of HepG2 cells during pulse–chase experiments performed with intact cells. In order to understand better the fate and subcellular trafficking of intravesicular free polymannose-type oligosaccharides in HepG2 cells we have initiated a series of experiments aimed at reconstituting the metabolism of these components in permeabilized cells.

ATP provokes the release of free polymannose oligosaccharides from permeabilized cells

In order to study the fate of free oligosaccharides in the vesicular compartment of the PCs we allowed these components to accumulate in this compartment by incubating the permeabilized cells as shown in Figure 1A (summarized in Figure 2A). PCs preincubated in the manner described above were washed twice in transport buffer and then incubated either in the absence or presence of ATP as described in Materials and methods. In the absence of ATP the quantity of free oligosaccharide observed in both the vesicular and cytosolic compartments of the PCs increased slightly (Figure 2B, left panel). In contrast, when PCs are incubated in the presence of ATP there is a rapid loss of oligosaccharides from the vesicular compartment of the PCs and a concurrent increase in oligosaccharide material recovered from the cytosolic compartment (Figure 2B, right panel).

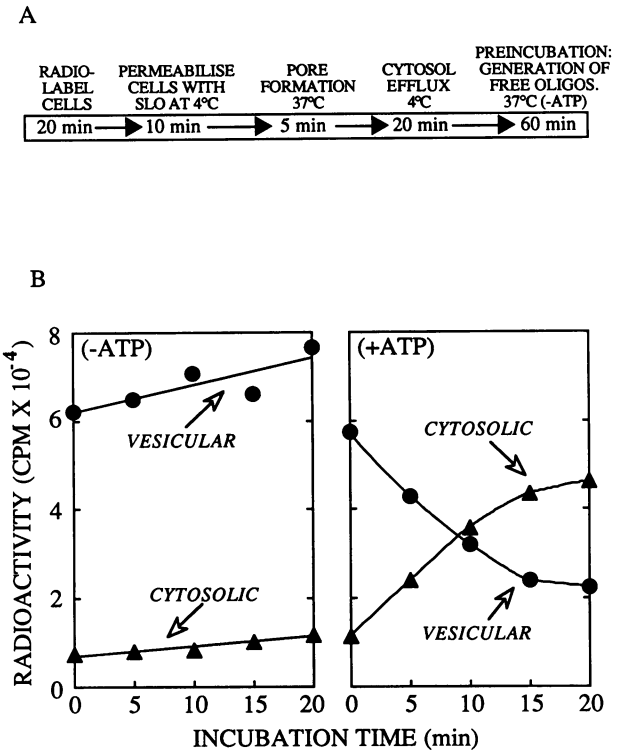

Fig. 2. The effect of ATP on the distribution of free oligosaccharides between the vesicular and cytosolic compartments of permeabilized cells. (**A**) A flow diagram summarizing the preparation of PCs, as described in Figure 1. (**B**) PCs, prepared as summarized above, were incubated at 37°C for the indicated times in either the absence (left panel) or presence (right panel) of ATP. After terminating the incubations neutral free oligosaccharides were isolated from both the incubation media, representing the cytosolic compartment (*CYTOSOLIC*), and permeabilized cells, representing the intravesicular compartment (*VESICULAR*), and quantitated by scintillation counting after charcoal chromatography. The values represent the total radioactivity recovered as neutral free oligosaccharides from each incubation.

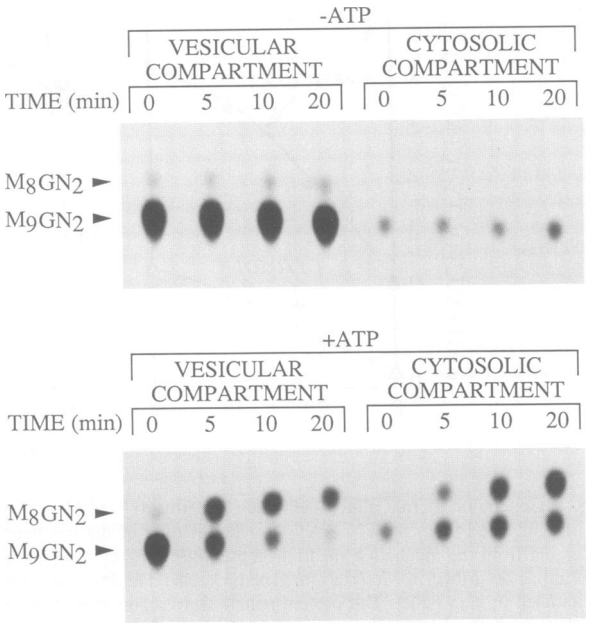

Fig. 3. Thin-layer chromatography of soluble free oligosaccharides isolated from the cytosolic and intravesicular compartments of control and ATP-treated PCs. PCs were incubated in either the absence (upper panel) or presence (lower panel) of ATP as described for Figure 2. The free oligosaccharides isolated from both the permeabilized cells (VESICULAR COMPARTMENT) and the incubation medium (CYTOSOLIC COMPARTMENT) were analysed by thin-layer chromatography as described in Materials and methods. The two observed oligosaccharide components have been indicated as M_8GN_2 and M_9GN_2 on account of their co-migration with standard $Man_8GlcNAc_1$ and $Man_9GlcNAc_1$, respectively, upon digestion with endo H.

Structural analysis of free oligosaccharides isolated from the intravesicular and cytosolic compartments of PCs after incubations performed in the absence or presence of ATP

Free oligosaccharides isolated from the experiment shown in Figure 2B were analysed by thin-layer chromatography as shown in Figure 3. Results demonstrate that in the absence of ATP the oligosaccharide material can be mainly accounted for by the species $Man_9GlcNAc_2$, and this component remains stable within the intravesicular compartment of the PCs. In contrast, when ATP is added to the incubations there is both a rapid conversion of $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ and efflux of both of these oligosaccharides into the cytosolic compartment. We confirmed that the oligosaccharide migrating as $Man_8GlcNAc_2$ was not $Man_9GlcNAc_1$ by investigating the nature of the reducing termini of the oligosaccharides isolated from incubations conducted in the presence of ATP. After derivatizing these components with 2-aminopyridine and digestion with endo H as described in Materials and methods we were able to show that all oligosaccharides generated in the presence of ATP terminated with the di-*N*-acetylchitobiose moiety (results not shown). Thus after preincubating permeabilized HepG2 cells in the absence of ATP to allow accumulation of free $Man_9GlcNAc_2$ in the vesicular compartment we show that after washing the PCs and re-addition of ATP there is an intravesicular conversion of free $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$, and a translocation of both these components into the cytosolic compartment of HepG2 cells.

The hydrolysis of ATP is required for the translocation of free oligosaccharides from the vesicular compartment to the cytosolic compartment of permeabilized HepG2 cells

Next we investigated the role of ATP in provoking the translocation of free oligosaccharides into the cytosolic compartment of permeabilized HepG2 cells. First, we showed that ATP has no effect on this translocation process if incubations are carried out at 4°C (results not shown). Figure 4A demonstrates that in the presence of the ATP-regenerating system alone there is no oligosaccharide translocation (0 mM ATP), that 0.5 mM ATP is sufficient to initiate the translocation process, and that between 5–10 mM ATP are required for maximal translocation. Furthermore, Figure 4B demonstrates that the non-hydrolysable ATP analogues AMP–PNP and AMP–PCP had no effect on the translocation process. In addition, Figure 4C shows that the effect of ATP on oligosaccharide translocation is largely dependent upon the inclusion of magnesium ions in the transport buffer.

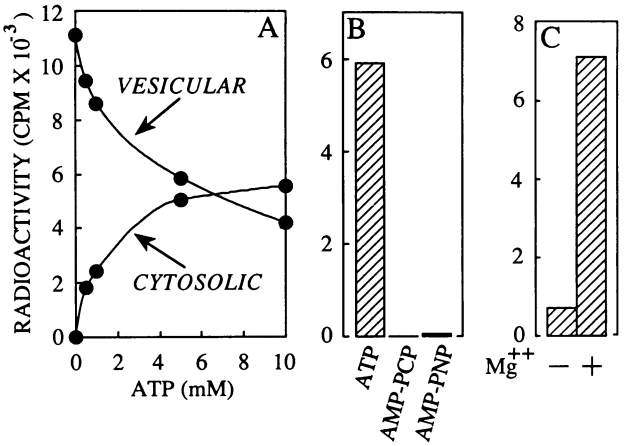

Fig. 4. The hydrolysis of ATP is required for the translocation of free oligosaccharides from the vesicular compartment to the cytosolic compartment of permeabilized HepG2 cells. (**A**) PCs were prepared as summarized in Figure 2A and then incubated in the presence of the ATP-regenerating system supplemented with different concentrations of ATP for 20 min at 37°C. After separating the PCs (intravesicular compartment, *VESICULAR*) from the incubation media (cytosolic compartment, *CYTOSOLIC*) by centrifugation, oligosaccharides were extracted from each fraction and assayed by scintillation counting after charcoal chromatography as described in Materials and methods. (**B**) In a different experiment PCs prepared as described above were incubated for 20 min at 37°C with either 5 mM ATP, 5 mM AMP-PCP or 5 mM AMP-PNP. Free oligosaccharides released into the incubation medium (cytosolic compartment) were quantitated by scintillation counting after charcoal chromatography. (**C**) PCs were prepared as above and then incubated for 20 min at 37°C under standard conditions (ATP-regenerating system supplemented with 7 mM ATP) in either the standard transport buffer (Mg^{2+}, +) or the standard transport buffer depleted of magnesium ions (Mg^{2+}, –); results are expressed as for panel B. In each experiment the oligosaccharide release occurring in the absence of ATP was subtracted from that occurring in the presence of the ATP analogues or that occurring with ATP under the different conditions.

ATP hydrolysis does not cause a generalized rupturing of ER membranes

In order to eliminate the possibility that ATP hydrolysis was increasing the permeability of ER membranes at 37°C in a non-specific manner we tested the selectivity of this transport process by loading HepG2 cells with free triglucosylated free polymannose oligosaccharides. This was achieved by pulse-radiolabelling intact cells for 20 min in the presence of the glucosidase inhibitor, castanospermine (CST) (Elbein, 1991) an agent that has been shown to severely reduce the appearance of OS-GN_2 in the cytosol of intact HepG2 cells (Moore and Spiro, 1994). After permeabilization of the CST-treated cells they were treated exactly as described in Figure 2A except that CST was included in the preincubation period. Results shown in Figure 5A clearly demonstrate that in CST-treated PCs (right panel) large quantities of oligosaccharide are generated in the intravesicular compartment of the PCs, but in comparison with control incubations (Figure 5A, left panel) there is no ATP-dependent release of oligosaccharide material into the cytosolic compartment. Thin-layer chromatography of the oligosaccharides generated in the presence of CST (Figure 5B) revealed that, as for unglucosylated oligosaccharides (Figure 3), there was an ATP-dependent intravesicular demannosylation, leading to the conversion of $Glc_3Man_9GlcNAc_2$ to $Glc_3Man_8GlcNAc_2$. Furthermore, when CST (1–10 mM) is added to

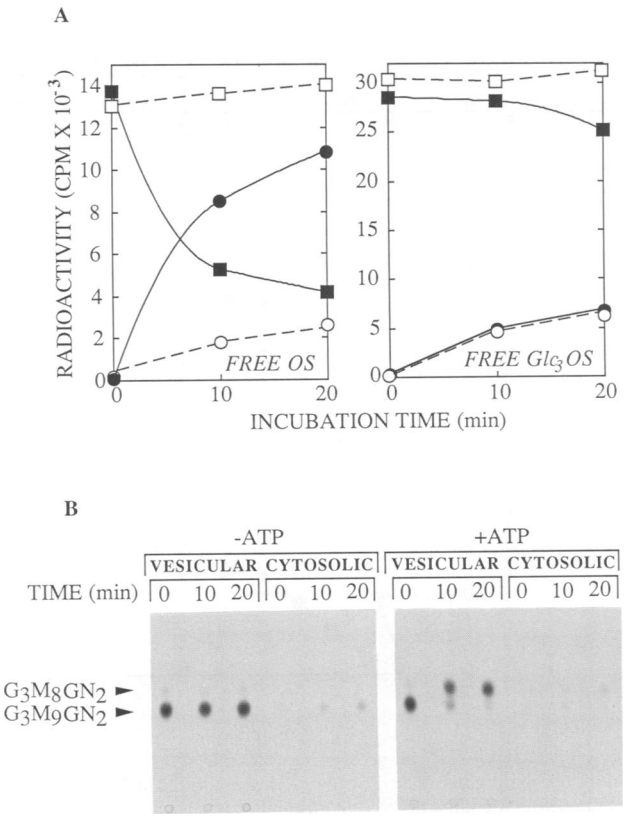

Fig. 5. Examination of the release of free triglucosylated oligosaccharides from the intravesicular compartment of control and ATP-treated PCs. (**A**) Permeabilized cells were prepared as summarized in Figure 2A except that, where indicated (right panel), before permeabilization HepG2 cells were pulse-radiolabelled for 20 min in the presence of 2 mM CST and thereafter all procedures conducted at 37°C were performed in the presence of the same concentration of this agent. Incubations were conducted in the presence (solid symbols) or absence (open symbols) of 7 mM ATP and an ATP-regenerating system. After terminating the incubations and separation of PCs (intravesicular compartment, squares) from the incubation media (cytosolic compartment, circles) neutral free oligosaccharides were isolated from each fraction as described in Materials and methods and quantitated by scintillation counting. (**B**) Thin-layer chromatography of the above isolated oligosaccharides from CST-treated PCs. The two observed oligosaccharide components have been indicated as $G_3M_8GN_2$ and $G_3M_9GN_2$ on acount of their co-migration with standard $Glc_3Man_8GlcNAc_1$ and $Glc_3Man_9GlcNAc_1$, respectively, upon digestion with endo H.

control PCs (prepared in the absence of CST) incubated in the presence of ATP we were unable to detect any inhibition of free oligosaccharide release, indicating that CST itself does not inhibit the transport process (results not shown). In order to verify that triglucosylated free polymannose oligosaccharides are freely soluble within the lumen of the ER we have treated PCs loaded with triglucosylated oligosaccharides with the detergent saponin. Figure 6 clearly demonstrates that there is no difference in the ability of saponin to release non-glucosylated versus their glucosylated counterparts from the intravesicular compartment of PCs. Accordingly, >95% of the soluble glucosylated oligosaccharides can be released from the vesicular compartment of saponin-treated PCs. In contrast, only 30% of radiolabelled glycoproteins could be released from the saponin-treated perme-

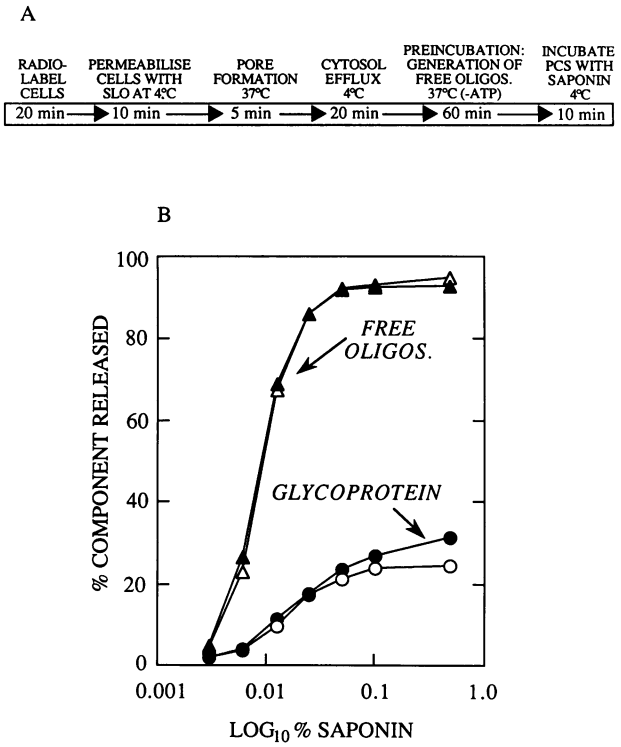

Fig. 6. Free triglucosylated oligosaccharides trapped within the intravesicular compartment of permeabilized cells can be released into the cytosolic compartment by low concentrations of saponin. (**A**) Summary of experimental protocol. PCs were prepared as summarized in Figure 2A, or when CST was used, modified as described in the legend to Figure 5. After the preincubation period the PCs were incubated for 10 min at 4°C with transport buffer containing different concentrations of saponin. (**B**) After separation of the PCs (intravesicular compartment) from the saponin-containing incubation media (cytosolic compartment). Free oligosaccharides (triangles) were isolated from each compartment and assayed by scintillation counting after charcoal chromatography. Glycoproteins (circles) were isolated from the intracellular compartment as described in Materials and methods, and after digestion with pronase were quantitated by scintillation counting. The closed symbols represent data from control PCs whereas the open symbols represent data obtained for the CST-treated PCs. For each component the quantity found in the cytosolic compartment was divided by the sum of the components found in both the cytosolic and vesicular compartments and has been expressed as a percentage.

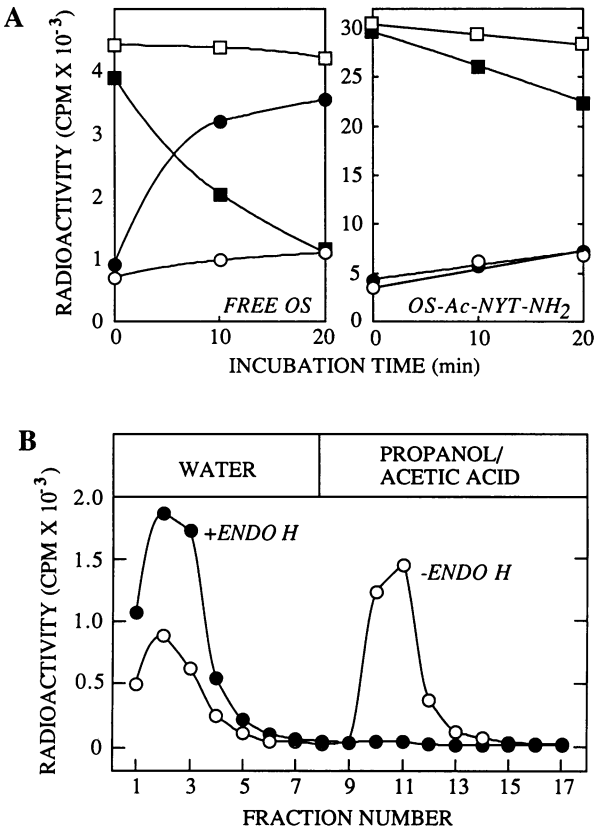

Fig. 7. Analysis of the fate of glycosylated Ac-NYT-NH_2 in permeabilized HepG2 cells. (**A**) PCs were prepared as summarized in Figure 2A except that during the preincubation period the PCs were incubated with 5 μM Ac-NYT-NH_2. PCs were re-incubated in the absence of Ac-NYT-NH_2 in either the absence (open symbols) or presence (closed symbols) of 7 mM ATP and the ATP-regenerating system. Oligosaccharides (*FREE-OS*) were separated from glycopeptides (*OS-Ac-NYT-NH_2*) on SepPak cartridges as described in Materials and methods and quantitated by scintillation counting [squares, PCs (intravesicular compartment); circles, incubation media (cytosolic compartment)]. (**B**) The medium (cytosolic compartment) from an incubation performed in the presence of ATP, similar to that shown in Figure 6A, was desalted by Biogel P6 chromatography and glycopeptide and oligosaccharide fractions were pooled, lyophilized, digested with endo H, and then resolved into free oligosaccharide and glycopeptide fractions by chromatography on SepPak cartridges as described in Materials and methods.

abilized cells, indicating that these components are either directly or indirectly attached to PC membranes.

Further evidence for the integrity of ER membranes upon incubation of PCs with ATP was obtained by loading PCs with the peptide, Ac-NYT-NH_2, which in intact cells has been shown to be glycosylated in the ER and exported to the exterior of the cell by vesicular transport (Wieland *et al.*, 1987). PCs were preloaded with glycopeptide by incubating them in the presence of Ac-NYT-NH_2 as described in Materials and methods. Subsequent incubations of PCs preloaded with glycopeptide revealed that whereas there was an efficient ATP-dependent release of the free oligosaccharides into the cytosolic compartment from the glycopeptide-loaded PCs (Figure 7A, left panel), the glycopeptide remained associated with the intravesicular compartment and there was no ATP-dependent increase in the recovery of glycopeptide from the cytosolic compartment (Figure 7A, right panel). In order to examine the structure of the glycopeptide that appeared in the cytosolic compartment of PCs the medium from a 20 min incubation, performed in the presence of ATP, was analysed by SepPak chromatography before and after digestion with endo H. Results (shown in Figure 7B) demonstrate that this glycopeptide material is completely endo H-sensitive, indicating that the small amounts of glycopeptide that are released into the cytosolic compartment do so without Golgi-type modification of their carbohydrate chains. Moreover, thin-layer chromatographic evaluation of the oligosaccharides released from intravesicular Ac-NYT-NH_2 by endo H revealed that, as observed for the non-glucosylated (Figure 3) and triglucosylated (Figure 5B) free oligosaccharides, the peptide-bound polymannose units underwent an intravesicular ATP-dependent demannosylation, yielding the structure $Man_8GlcNAc_2$-N(Ac)YT-NH_2 from $Man_9GlcNAc_2$-N(Ac)YT-NH_2 (results not shown).

Finally, we investigated the effect of demannosylation

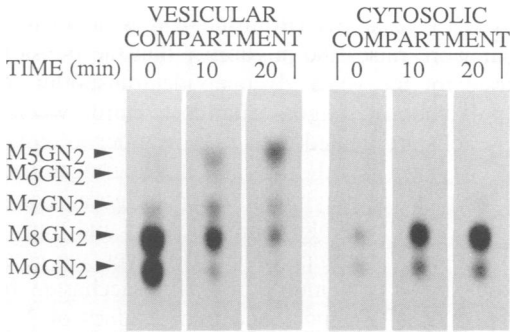

Fig. 8. Analysis of the free oligosaccharides derived from incubations of permeabilized cells prepared from BFA-treated intact HepG2 cells. HepG2 cells pretreated with 10 μg/ml BFA for 45 min were pulse-radiolabelled for 20 min and permeabilized with SLO as described in Materials and methods and summarized in Figure 2A. The resulting PCs were incubated for the indicated times with 7 mM ATP and the ATP-regenerating system in exactly the same manner to that described in Figure 2. After separating the PCs (VESICULAR COMPARTMENT) from the incubation media (CYTOSOLIC COMPARTMENT) free oligosaccharides were prepared from each compartment and analysed by thin-layer chromatography after charcoal chromatography as described in Materials and methods. The observed oligosaccharide components have been indicated as M_9GN_2, M_8GN_2, M_7GN_2, M_6GN_2 and M_5GN_2 on account of their co-migration with standard $Man_9GlcNAc_1$, $Man_8GlcNAc_1$, $Man_7GlcNAc_1$, $Man_6GlcNAc_1$ and $Man_5GlcNAc_1$, respectively, upon digestion with endo H.

of intravesicular free polymannose oligosaccharides on their ability to be translocated into the cytosol. In order to achieve this we pretreated intact HepG2 cells with brefeldin A (BFA) in order to redistribute Golgi enzymes into the ER (Lippincott-Schwartz *et al.*, 1989). Subsequent to permeabilization of the cells as described in Materials and methods immunofluoresence studies (results not shown) using an anti-galactosyltransferase antibody revealed the presence of staining characteristic of the Golgi apparatus in control PCs, whereas in the PCs prepared from BFA-treated cells the staining was more diffuse, confirming that the BFA had caused the redistribution of this marker enzyme into the ER (Berger *et al.*, 1993). In PCs pretreated with BFA we were unable to detect any free oligosaccharide processing unless ATP was added to the incubation mixtures (results not shown). Figure 8 demonstrates that addition of ATP to the PCs provokes two phenomena; first, there is a rapid intravesicular demannosylation of free polymannose-type oligosaccharides to yield the component $Man_5GlcNAc_2$, and second, there is a rapid release of $Man_{9-8}GlcNAc_2$ oligosaccharides into the cytosolic compartment. Although longer exposures of the thin-layer chromatography fluorographs did reveal small amounts of $Man_5GlcNAc_2$ in the cytosolic compartment of the PCs, it is evident that after 20 min incubations in the presence of ATP this component is localized predominantly in the intravesicular compartment, further demonstrating that ATP treatment of PCs does not lead to general breakdown of ER membranes.

Discussion

In the present study we have used permeabilized HepG2 cells in order to demonstrate the transport of free oligosaccharides from an intravesicular compartment directly into the cytosol. The intravesicular compartment from which this transport process occurs is likely to be the ER for the following reasons. As we show that the quantity of glycoprotein remains stable during incubations of PCs and because protein biosynthesis is unlikely to occur under these conditions [a process requiring both GTP and ATP (Rapoport, 1992)] it leaves oligosaccharide–lipid as the only possible source of free polymannose oligosaccharides. In accordance with this the increase in radioactivity associated with the free oligosaccharides generated during the preincubation period could be accounted for by the observed decrease in the radioactivity associated with oligosaccharide–lipid (Figure 1A and B). Thus, the oligosaccharides terminating at their reducing end with a di-*N*-acetylchitobiose moiety ($OS\text{-}GN_2$) observed here are, as others have previously shown (Anumula and Spiro, 1983; Spiro and Spiro, 1991; Villers *et al.*, 1994), derived from dolichol-PP–oligosaccharides which are synthesized in the lumen of the ER (Hirschberg and Snider, 1987). In agreement with the notion that $OS\text{-}GN_2$ arise from oligosaccharide–lipid in the PCs is the fact that a substantial proportion of these free oligosaccharides are glucosylated at the outset of the preincubation period. However, during the preincubation period these components are rapidly deglucosylated, even in the absence of ATP. Furthermore, when PCs were incubated with Ac-NYT-NH_2 this peptide became glycosylated, causing an almost complete loss of radioactivity associated with oligosaccharide–lipid (results not shown, compare this situation with that observed in the absence of peptide where substantial amounts of oligosaccharide–lipid remain; Figure 1B). Accordingly, a comparison of the left panel of Figure 5A with the left panel of Figure 7A (data from these two experiments were obtained at the same time using the same batch of PCs) reveals that when PCs are preincubated in the presence of Ac-NYT-NH_2 there is a 3-fold reduction in the quantity of free polymannose-type oligosaccharides. Presumably the residual free oligosaccharides observed in the Ac-NYT-NH_2-treated PCs represent free oligosaccharides already present in the intravesicular compartment of PCs at the beginning of the preincubation period (see Figure 1A). These results suggest that peptide glycosylation mediated by oligosaccharyltransferase and free oligosaccharide generation occur in the same compartment. In addition, further evidence that these free oligosaccharides reside in the lumen of the ER is demonstrated by the stability of oligosaccharide material observed in the vesicular compartment of PCs incubated in the absence of ATP and the very limited intravesicular oligosaccharide processing that is apparent in the presence of ATP. Indeed, the conversion of $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ is a reaction known to be carried out by the ER mannosidase (Bischoff, 1986). The ATP-dependent removal of a single mannose residue from $Man_9GlcNAc_2$ to yield $Man_8GlcNAc_2$ in permeabilized cells was unexpected, as it has been previously shown that the *in vivo* conversion of glycoproteins bearing $Man_9GlcNAc_1$ to those bearing $Man_8GlcNAc_1$ can occur under energy-depleting conditions (Godelaine *et al.*, 1981).

In addition to demonstrating that free polymannose oligosaccharides are generated in the lumen of the ER we have also provided evidence that there is no vesicular transport in our PCs. Accordingly, we were only able to detect Golgi-type demannosylation of free oligo-

saccharides in the vesicular compartment of PCs if the intact cells from which the PCs were derived had been treated with BFA. This result was underlined by the fact that under the conditions suitable for the export of free oligosaccharides from ER to cytosol there was no evidence that glycoproteins acquired oligosaccharide structures consistent with their migration from the ER into the Golgi apparatus by vesicular transport (results not shown).

Thus, upon addition of ATP to the PCs our results demonstrate that OS-GN_2 are rapidly transported from the lumen of the ER into the cytosolic compartment of permeabilized HepG2 cells. We have noted variability in the rate and extent of the ATP-provoked transport of free oligosaccharides; under optimal conditions the release of oligosaccharides into the cytosolic compartment was 50–80% complete. At present, the origin of this variability is unknown but might perhaps be due to the between-batch variations in potency of the hexokinase used in the ATP degeneration system utilized during the preincubations. If small amounts of hexokinase rest with the PCs during the transport assay this may reduce the efficiency of the observed transport. Although 0.5 mM ATP was sufficient to provoke oligosaccharide translocation in our test system, supraphysiological levels of ATP were required in order to elicit maximal translocation. Indeed, the ATP requirement for oligosaccharide transport demonstrated here is very similar to that required to observe maximal peptide transport into the ER by the TAP1/TAP2 ATPase (Neefjes *et al.*, 1993).

In order to demonstrate a transport process for the translocation of free oligosaccharides from the lumen of the ER into the cytosol we had to demonstrate that ATP hydrolysis was not simply reducing the integrity of the ER membranes. To this end we have provided three different instances whereby ATP hydrolysis is incapable of provoking the release of small molecules from the vesicular compartment of PCs. First, membrane integrity was evaluated by performing experiments on PCs that had been primed with triglucosylated oligosaccharides. Under these conditions ATP caused the rapid intravesicular conversion of $Glc_3Man_9GlcNAc_2$ to $Glc_3Man_8GlcNAc_2$ but failed to provoke the release of these components into the cytosolic compartment, despite the fact that saponin treatment of such PCs caused >95% of these triglucosylated components to be recovered in the cytosolic compartment. Second, when PCs were preloaded with glycotripeptide, ATP was unable to provoke the release of this component into the cytosolic compartment. In mammalian cells, including HepG2, glycosylated Ac-NYT-NH_2 has been shown to be a marker for the bulk flow of material from the ER to cell surface by vesicular transport (Wieland *et al.*, 1987) and in accordance with this it has been demonstrated that mammalian ER does not have the ability to export glycotripeptide from the ER into the cytosol (van Leyen *et al.*, 1994). The rate of ATP-dependent oligosaccharide transport out of the ER, observed in the present report, was similar to that observed for the transport of iodinated glycotripeptide from the ER of broken yeast cells (Römisch and Schekman, 1992); both processes occur without a lag period and are half maximal between 5 and 15 min. In the present study we have not directly addressed the question of whether there is a mammalian glycopeptide transporter similar to that observed in yeast ER (Römisch and Schekman, 1992), but our work raises the possibility that the physiological substrate for the yeast glycopeptide transporter may be free polymannose oligosaccharides. Third, when intact HepG2 cells are treated with BFA in order to redistribute Golgi enzymes into the ER and then permeabilized, we observed that free polymannose-type oligosaccharides are both processed by Golgi mannosidase I and translocated into the cytosolic compartment. However, we noted that an extensively demannosylated oligosaccharide bearing five residues of mannose and two residues of *N*-acetylglucosamine resisted export from the vesicular compartment of the PCs. In summary, our results demonstrate that ATP hydrolysis does not reduce the integrity of ER membranes.

Why does ATP lead to a specific translocation of free oligosaccharides from the lumen of the ER into the cytosolic compartment? One hypothesis is that the ER membrane contains a transport molecule coupled to an ATPase (Higgins, 1992), and the specificity of the observed transport process reflects the substrate specificity of this transporter. However, other more complex hypotheses for this ATP-provoked ER-to-cytosol transport should also be considered. We are currently investigating the role of ATP in this transport process.

Whatever the mechanism of the ATP-provoked ER-to-cytosol oligosaccharide transport in permeabilized HepG2 cells, the metabolism of free oligosaccharides observed in the PCs shows similarities to that observed in intact HepG2 cells. Accordingly, we have failed to observe cytosolic glycosylated Ac-NYT-NH_2 in the cytosol of intact HepG2 cells, despite noting its appearance in both the vesicular compartment and in the incubation media (S.E.H.Moore and P.Codogno, unpublished observations). Furthermore, when intact HepG2 cells are radiolabelled in the presence of CST and chased in the presence of this inhibitor, only very small quantities of OS-GN_2 are recovered in the cytosolic compartment of intact HepG2 cells (Moore and Spiro, 1994).

At present, the reason for the release of free oligosaccharides into the lumen of the ER is unclear but it is apparent that the cell invests considerable effort in segregating these components from those following the secretory pathway. As it is now known that free oligosaccharides can interact with calnexin *in vitro* (Ware *et al.*, 1995) and can be substrates for Golgi mannosidase I (this work), the rapid transport of free oligosaccharides from the lumen of endoplasmic reticulum into the cytosol may ensure that these components do not interfere with the folding, processing and trafficking of glycoproteins along the secretory pathway. Although in the presently described permeabilized cell system oligosaccharides are apparently stable in the cytosolic compartment, it is known that *in vivo* the cytosol contains both an endo H-like enzyme (Pierce *et al.*, 1979) and an α-mannosidase (Tulsiani and Touster, 1987; Grard *et al.*, 1994) which are capable of acting, *in vivo*, upon cytosolic OS-GN_2 to yield the linear isomer of $Man_5GlcNAc_1$ (Moore and Spiro, 1994). It has been speculated that the accumulation of this oligosaccharide in the cytosol is restricted by its translocation into and degradation in the lysosome (Tulsiani and Touster, 1987; Moore and Spiro, 1994). A corollary of the dual fate of glycoproteins and free oligosaccharides is that

glycoproteins and not free oligosaccharides are the sole extracellular carriers of information encoded in oligosaccharide structures.

Our observations demonstrate for the first time the presence of a transport process that enables free oligosaccharides to escape the vesicular transport pathway and gain access to the cytosol. Further studies are being conducted in order to investigate the regulation of free oligosaccharide production in the lumen of the endoplasmic reticulum and to characterize the endoplasmic reticulum-to-cytosol transport process.

Materials and methods

Growth and radiolabelling of cells

HepG2 cells (ECACC, Porton Down, UK) were cultivated as previously described (Moore and Spiro, 1990). For the preparation of radiolabelled permeabilized cells, confluent 75 cm^2 flasks were incubated with 200 μCi D-[2-^{3}H]mannose (25 Ci/mmol; ICN, Irvine, CA) for 20 min in 2 ml glucose-free Dulbecco's modified Eagle's medium supplemented with 5% dialysed fetal calf serum, 2 mM glutamine and 1 mM sodium pyruvate. Where indicated, cells were preincubated with CST (2 mM, Cambridge Research Biochemicals, Northwich, UK) or BFA (10 μg/ml, Boehringer-Mannheim, Germany) in radiolabelling medium for 45 min before the addition of radiolabel.

Preparation of permeabilized cells

Subsequent to pulse-radiolabelling, cells were washed twice with ice-cold PBS containing 1 mM EDTA and then released from the tissue culture flask by incubation with 2 ml prewarmed trypsin/EDTA for 1 min at 37°C. After addition of 10 ml ice-cold complete growth medium the cells were harvested and washed twice with the transport buffer (Neefjes *et al.*, 1993); 130 mM K^+/glutamate, 10 mM NaCl, 2 mM EGTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM HEPES–KOH, pH 7.3, by centrifugation at 500 g_{Av}. Cells were then permeabilized for 10 min at 4°C in 5 ml transport buffer containing 1 U/ml SLO (Murex, Dartford, UK), then after washing the cells twice at 4°C in transport buffer pore formation was completed by addition of 2 ml prewarmed transport buffer (containing 2 mM CST or 10 μg/ml BFA where indicated) and incubating the cells at 37°C for 5 min. After rapidly cooling the cells by the addition of ice-cold transport buffer they were incubated for 20 min on ice to allow further efflux of cytosolic components.

Priming of permeabilized HepG2 cells with free oligosaccharide

PCs were incubated in transport buffer at 37°C for 60 min in the presence of an ATP-degenerating system (5 mM mannose and 40 IU hexokinase, Colombo *et al.*, 1992) and 20 μM each of GDP-mannose, UDP-glucose and UDP-*N*-acetylglucosamine. PCs from one confluent 75 cm^2 flask were incubated in 1 ml of this mixture. For loading of PCs with glycopeptide the preincubation mixture was supplemented with 5 μM Ac-NYT-NH_2 (synthesized and purified according to Wieland *et al.*, 1987 by Neosystem, Strasbourg, France). CST or BFA were included in the preincubation mixture, where appropriate, at a concentration of 2 mM and 10 μg/ml, respectively. PCs derived in the above-described manner were used for between 10 and 20 incubations as described below.

Oligosaccharide transport assay in permeabilized HepG2 cells

After terminating the preincubations the PCs were washed twice at 4°C in transport buffer and then incubated in this buffer supplemented, where indicated, with the following: ATP and an ATP-regenerating system (7 mM ATP, 3.5 mM creatine phosphate, 25 mg/ml creatine phosphokinase), the ATP-degenerating system and 2 mM CST, in a final volume of 50 μl. Incubations were terminated by the addition of 450 μl cold transport buffer and after a 10 min incubation on ice the PCs were separated from the incubation media by centrifugation. Where indicated the transport assays were stopped by the addition of ice-cold transport buffer containing 0.05% saponin and thereafter treated as described above.

Isolation of oligosaccharides and glycopeptides

The procedures for the isolation of oligosaccharides from the intravesicular compartment of PCs and SLO-perfusates (here called 'cytosolic compartment') have been described in detail previously (Moore and Spiro, 1994). When the peptide Ac-NYT-NH_2 was incubated with PCs, free oligosaccharides and glycopeptides were isolated in identical fashion, except that after desalting of the crude oligosaccharide fractions the water eluates were passed directly through SepPak columns (Waters, Millipore Corporation) before charcoal chromatography (Moore and Spiro, 1990). Free oligosaccharides were recovered from the reversed phase cartridges in the effluent and water washes and subjected to charcoal chromatography as described (Moore and Spiro, 1990) whereas glycopeptides were eluted with 20% *n*-propanol in 1 M acetic acid.

Structural analysis of oligosaccharides and glycopeptides

OS-GN_2 and OS-GN_1 oligosaccharides were separated as previously described (Moore and Spiro, 1994) after derivatization with 2-aminopyridine. Upon subsequent treatment of derivatized oligosaccharides with endo H, and resolution of the digestion products on columns AG 50-X2 (H^+ form), endo H-sensitive oligosaccharyl–aminopyridines were recovered as their OS-GN_1 counterparts in the water effluent whereas endo H-resistant oligosaccharyl–aminopyridines were eluted from the AG-50 with 0.2 M ammonium hydroxide. Oligosaccharide structures attached to Ac-NYT-NH_2 were released from the peptide by endo H and the completeness of the digestions was assessed by chromatography of the incubation mixtures on SepPak columns as described above. Free oligosaccharides passing through the SepPak columns were desalted on combined columns of AG 50-X2 (H^+ form) over AG 1-X2 (acetate form) before thin-layer chromatography. In some cases the incubation media were directly applied to a column (1×100 cm) of Biogel P6 equilibrated with 0.1 M pyridine acetate, pH 5.0, and the radioactive components migrating between V_0 and V_i were pooled, lyophilized and digested with endo H as described above.

Isolation of oligosaccharide–lipids and glycoproteins from permeabilized cells

These methods have been extensively described elsewhere (Spiro *et al.*, 1976; Moore and Spiro, 1990). Briefly, after PCs were extracted with chloroform:methanol:water (3:2:1), the protein pellet was washed once with water and then once with methanol before being extracted with chloroform:methanol:water (10:10:3), to yield an oligosaccharide–lipid fraction and a delipidated protein pellet. Oligosaccharide–lipids were quantitated by scintillation counting, whereas the protein pellet was digested with pronase before quantitation in the same manner. The resulting glycopeptides were digested with endo H as previously described (Moore and Spiro, 1990) and after desalting of the released polymannose units on combined columns of AG-1 and AG-50 they were resolved by thin-layer chromatography as described below.

Thin-layer chromatography

Free oligosaccharides were analysed on plastic thin-layer chromatography plates coated with silica (Merck) and developed in *n*-propanol:acetic acid:water (3:3:2) for 20 h. Resolved oligosaccharides were visualized on Hyperfilm-MP (Amersham France SA) by fluorography. Standard oligosaccharides were prepared from HepG2 cells as previously described (Moore and Spiro, 1994).

Immunofluorescence microscopy

Permeabilized cells were prepared as described above from either BFA-treated (10 μg/ml for 45 min) or control intact cells. The resulting PCs were fixed with 0.1% paraformaldehyde and then repermeabilized with 0.075% saponin in PBS for 20 min before staining with a monoclonal antibody directed against human milk galactosyltransferase GT2/36/118 (diluted 1:1) (Berger *et al.*, 1986) and then secondary staining with an FITC-conjugated rabbit anti-mouse immunoglobulin (diluted 1:200, Jackson ImmunoResearch, West Grove, PA). Cell labelling was visualized using a Zeiss Axioplan photomicroscope equipped for epifluorescence. Controls without primary antibody were also included.

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