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Glycopeptide export from mammalian microsomes is independent of calcium and is distinct from oligosaccharide export

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Glycopeptides are exported from the endoplasmic reticulum to the cytosol of eukaryotic membranes in an ATP- and cytosol-requiring process (Romisch and Ali, 1997, *Proc. Natl. Acad. Sci. USA*, 94, 6730–6734). Oligosaccharides of the polymannose-type are also exported from the endoplasmic reticulum of mammalian cells to the cytosol in an ATP-dependent fashion. These findings raise the strong possibility that the two substrate classes are transported by the same mechanism but the precise identity of the translocation machinery for each substrate class has not been fully defined. Here we have investigated the mechanism by which a glycopeptide is exported from rat liver microsomes, and compare this to the export of free polymannose oligosaccharides. Using EGTA and the endoplasmic reticulum calcium mobilizing agents thapsigargicin and calcium ionophores A23187 and ionomycin, we show that glycopeptides, in contrast to oligosaccharides, are exported by a calcium-independent mechanism. On the other hand, Mg^{2+} is required in the assay for the transport of glycopeptide from mammalian microsomes which is in common with oligosaccharide export. Deoxynojirimycin and castanospermine, inhibitors of ER glucosidases, when added to rat liver microsomes prior to loading with peptide that bears an *N*-glycosylation sequon, had no effect on the release of glucosylated glycopeptides from membranes, indicating that removal of the α-glucose units from the oligomannose glycan structure of the glycopeptide is not required for export. In contrast to oligosaccharides, where transport is efficiently inhibited, mannosides were without effect or only weak inhibitors of glycopeptide export. Taken together, these data suggest that glycopeptides are exported by a distinct mechanism from oligosaccharides of the polymannose-type and that the peptide moiety is an important structural determinant for glycopeptide export and capable of directing translocation of substrates to a specific transport pathway.

Key words: glycopeptide/oligosaccharide/endoplasmic reticulum/ERAD/transport

Introduction

Glycopeptide export from the endoplasmic reticulum (ER) to the cytosol is a conserved process in eukaryotes and has been demonstrated in both yeast and mammalian systems (Romisch and Schekman, 1992; Romisch and Ali, 1997). The export process is dependent on cytosol, requires ATP-hydrolysis and physiological temperature and can be achieved using heterologous sources for ER membranes and cytosol (Romisch and Ali, 1997). The export system is presumably part of the endoplasmic reticulum quality control machinery which allows only properly folded and fully assembled proteins to be transported from ER to the Golgi complex via the vesicular transport route (Hammond and Helenius, 1995). Potentially, allowing glycopeptides or oligopeptides into the Golgi complex or other post-ER secretory pathway compartments could lead to competition with true secretory cargo for the various modification and trafficking processes, and hence lead to deleterious consequences for the cell. Most recently, misfolded proteins have also been found, unexpectedly, to be exported out of the ER via the Sec61p translocon complex to the cytosol for degradation by the proteasome, indicating a bidirectional movement of proteins across the ER membrane (Brodsky and McCracken, 1997; Kopito, 1997; Bonifacino and Weissman, 1998; Suzuki *et al.*, 1998; Wilbourn *et al.*, 1998). At the present time, it is unclear if the export of misfolded proteins and glycopeptides are mediated by the same mechanism, or if more than one distinct route for retranslocation is present in the ER.

Free oligosaccharides of the polymannose-type are known to be generated in the lumen of the ER as a byproduct of glycoprotein biosynthesis (Anumula and Spiro, 1983; Cacan *et al.*, 1987) and, as in the case of misfolded proteins and glycopeptides, are expected to be removed from the secretory pathway as early as possible to prevent competition with glycoprotein processing events. Indeed, using a HepG2 cell system, Moore and co-workers demonstrated that oligomannose oligosaccharides are efficiently released from the ER into the cytosolic compartment (Moore and Spiro, 1994; Moore *et al.*, 1995). Interestingly, these oligosaccharides are trimmed in the cytosol and then translocated into lysosomes for further degradation (Saint-Pol *et al.*, 1997).

Although there are several differences between glycopeptide and free polymannose oligosaccharides export systems, both show a requirement for ATP hydrolysis (Romisch and Schekman, 1992; Romisch and Ali, 1997; Moore *et al.*, 1995), which raises the possibility that the two substrates may be exported from the ER by the same or highly related transport mechanisms. Recently, Romisch and Ali (1997) reported the export of glycopeptide from rat liver microsomes and Moore

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(1998) reported further biochemical studies on the polymannose oligosaccharides export from permeabilized HepG2 cells which allowed comparison of the requirements of the two processes. Moore (1998) has shown a requirement for a thapsigargin-sensitive Ca^{2+} store for the export of polymannose oligosaccharides to take place and also showed that export can be potently inhibited by various mannosides. We carried out glycopeptide export from rat liver microsomes in the presence of calcium ionophores and thapsigargicin, an analogue of thapsigargin, and found in contrast to oligosaccharides export, that these compounds have stimulatory effects on glycopeptide export from rat liver microsomes indicating that ER lumenal Ca^{2+} is not required. In addition Mg^{2+} but not Ca^{2+} was required in the assay buffer for export to take place from rat liver microsomes. We also tested a panel of mannosides and found little or no inhibitory effects on glycopeptide export. These data, together with results from previous studies, indicate that glycopeptides and free polymannose oligosaccharides are exported from ER to the cytosol by distinct mechanisms.

Results

Magnesium but not calcium is required for glycopeptide export from the ER of rat liver microsomes

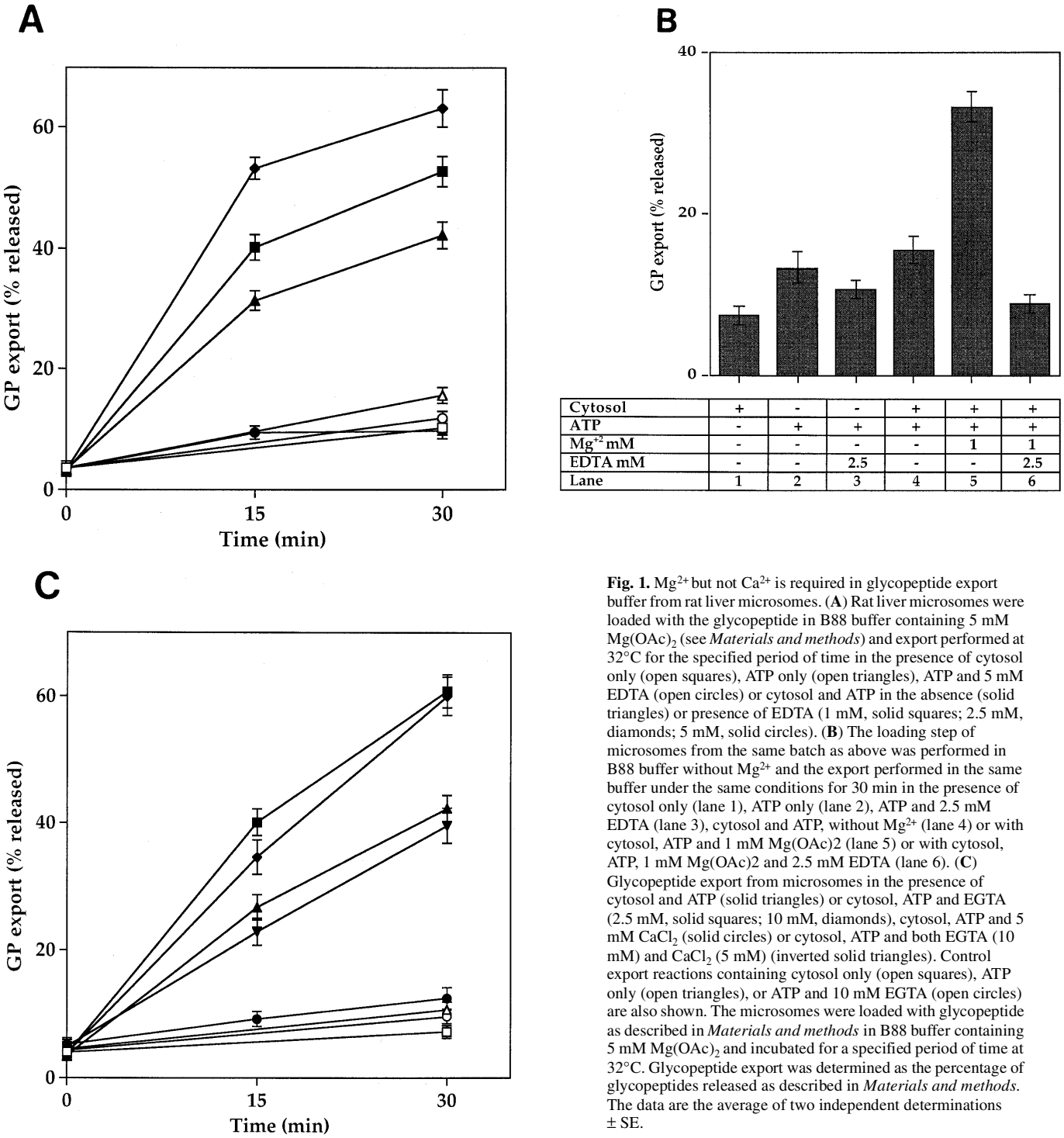
The glycopeptide export assay is based on introducing a hydrophobic iodinated tripeptide (Ac-NYT-NH_2), which contains the sequon for N-glycosylation, into ER membranes. Once inside the ER, the peptide is then glycosylated by oligosaccharyltransferase and becomes both larger in mass and more hydrophilic and therefore can not exit the ER without the addition of ATP, cytosol and incubation at physiological temperature. Glycopeptides in both supernatant and membrane fractions are then quantified by capture with concanavalin A (ConA) Sepharose followed by γ-counting. The export conditions we used are based on systems designed to reconstitute, *in vitro*, ER to Golgi vesicular transport (Baker *et al.*, 1988) and which were also found to promote export of glycopeptide, critically by a nonvesicular mechanism, from yeast ER to the cytosol (Romisch and Schekman, 1992). In contrast to an earlier report (van Leyen *et al.*, 1994), these conditions also support glycopeptide export from mammalian microsomes (Romisch and Ali, 1997). Mg^{2+}, in the form of magnesium acetate, is normally present at 5 mM in the assay mix. To test the requirement for this metal cation, we manipulated the concentration by adding increasing amounts of EDTA (Figure 1A). In the presence of ATP and an ATP-regenerating mix (referred to simply as ATP hereafter) and partially purified rat liver cytosol (similarly cytosol hereafter), EDTA at concentrations below 5 mM stimulated export of glycopeptide (compare solid squares and diamonds with solid triangles). At equimolar concentration to Mg^{2+}, i.e., 5 mM (solid circles), or above (not shown), EDTA inhibited the export activity to background levels. EDTA at 5 mM on its own or in the presence of ATP (not shown) or cytosol (open circles) has no effect on membrane integrity as released glycopeptide is similar to controls (cytosol only, open squares or ATP only, open triangles). The stimulation at low concentrations of EDTA is presumably a result of either the removal of cations that interfere with the export or alteration to the phospholipid structure of ER membrane to make export more efficient. The loading step of membranes with glycopeptide used for this experiment, and also in routine assays, was performed in B88 buffer which contains 5 mM Mg^{2+} (see *Materials and methods*). Therefore, to test the effect of complete absence of Mg^{2+}, we loaded membranes, from the same batch used in Figure 1A, in B88 buffer without Mg^{2+} and carried out the export reaction in the presence or absence of Mg^{2+} or EDTA. The results (Figure 1B) confirm the requirement for Mg^{2+} in the assay mix (compare lanes 4 vs. 5 and 6). Control export reactions (cytosol only, lane 1; ATP only, lane 2 or ATP and EDTA, lane 3) for these membranes were similar to membranes loaded under standard conditions (Figure 1A) indicating that membrane integrity was not compromised.

Export of oligosaccharides of the polymannose-type from HepG2 membranes requires calcium in the assay mixture (Moore *et al.*, 1995; Moore, 1998), hence we tested the effects of addition of $CaCl_2$ to the assay. Unexpectedly, we found the cation to be inhibitory to glycopeptide export at 5 mM (Figure 1C, solid triangles vs. solid circles for complete reactions without or with 5 mM $CaCl_2$, respectively). EGTA, a Ca^{2+} chelator stimulated the export slightly at 2.5 and 10 mM (solid diamonds and squares, respectively, vs. solid triangles). Critically, EGTA at 10 mM relieved the inhibitory effects of 5 mM Ca^{2+} (compare solid circles vs. inverted solid triangles) indicating that Ca^{2+} is indeed an inhibitor. All these data clearly show that Ca^{2+} is not required in the assay mix for the export to take place whereas Mg^{2+} is needed. Zn^{2+} and Mn^{2+} can not replace Mg^{2+} and in fact they inhibited the export completely at 2 and 5 mM, respectively (Table I). Including additional amounts of salts ($CaCl_2$, $MgCl_2$, $Mg(OAc)_2$, KCl, or NaCl) in the assay mixture resulted in reduced export of glycopeptide

Table I. Effects of salts on glycopeptide export from rat liver microsomes

Salt	Concentration [mM]	Export (% of control)
None	—	100 ± 5.2
$ZnCl_2$	2	-5.4 ± 1.0
	5	-6.0 ±1.2
$MnCl_2$	5	-3.9 ± 1.0
	25	-3.5 ± 0.8
$CaCl_2$	5	21.9 ± 2.2
	25	-4.9 ± 0.8
$MgCl_2$	5	57 ± 9.8
	25	9.6 ± 0.8
$Mg(OAc)_2$	50	12.0 ± 2.1
KCl	50	44.0 ± 3.9
	200	17.9 ± 2.4
NaCl	50	57.0 ± 4.2
	200	28.7 ± 2.3

Salts were added to export stage of the assay and incubations were at 32°C for 20 min. The percentage of released glycopeptide was determined as described in *Materials and methods*. Background levels (ATP only) were subtracted from each sample and then normalized to sample with ATP and cytosol in B88 buffer (100%). Values are the averaged of two independent determinations ± SE.



Cytosol	+	-	-	+	+	+
ATP	-	+	+	+	+	+
Mg^{+2} mM	-	-	-	-	1	1
EDTA mM	-	-	2.5	-	-	2.5
Lane	1	2	3	4	5	6

Fig. 1. Mg^{2+} but not Ca^{2+} is required in glycopeptide export buffer from rat liver microsomes. (**A**) Rat liver microsomes were loaded with the glycopeptide in B88 buffer containing 5 mM $Mg(OAc)_2$ (see *Materials and methods*) and export performed at 32°C for the specified period of time in the presence of cytosol only (open squares), ATP only (open triangles), ATP and 5 mM EDTA (open circles) or cytosol and ATP in the absence (solid triangles) or presence of EDTA (1 mM, solid squares; 2.5 mM, diamonds; 5 mM, solid circles). (**B**) The loading step of microsomes from the same batch as above was performed in B88 buffer without Mg^{2+} and the export performed in the same buffer under the same conditions for 30 min in the presence of cytosol only (lane 1), ATP only (lane 2), ATP and 2.5 mM EDTA (lane 3), cytosol and ATP, without Mg^{2+} (lane 4) or with cytosol, ATP and 1 mM Mg(OAc)2 (lane 5) or with cytosol, ATP, 1 mM Mg(OAc)2 and 2.5 mM EDTA (lane 6). (**C**) Glycopeptide export from microsomes in the presence of cytosol and ATP (solid triangles) or cytosol, ATP and EGTA (2.5 mM, solid squares; 10 mM, diamonds), cytosol, ATP and 5 mM $CaCl_2$ (solid circles) or cytosol, ATP and both EGTA (10 mM) and $CaCl_2$ (5 mM) (inverted solid triangles). Control export reactions containing cytosol only (open squares), ATP only (open triangles), or ATP and 10 mM EGTA (open circles) are also shown. The microsomes were loaded with glycopeptide as described in *Materials and methods* in B88 buffer containing 5 mM $Mg(OAc)_2$ and incubated for a specified period of time at 32°C. Glycopeptide export was determined as the percentage of glycopeptides released as described in *Materials and methods*. The data are the average of two independent determinations ± SE.

from rat liver membranes (Table I) indicating a requirement for physiological ionic strength for the export to take place.

Thapsigargicin and calcium ionophores stimulate glycopeptide export

We next asked if ER lumenal Ca^{2+} is required, as opposed to cytoplasmic Ca^{2+} for glycopeptide export. To achieve this, we utilized several compounds that alter Ca^{2+} concentrations in the ER by differing mechanisms. Firstly, we tested the effect of thapsigargicin, a less toxic analog of thapsigargin, which depletes ER lumenal Ca^{2+} by potent inhibition of the ER-Ca^{2+}ATPase (Lytton *et al.*, 1991). At 25 nM and higher, thapsigargicin stimulated glycopeptide export by ~25% (Figure 2A). Thapsigargicin, when present at up to 500 nM in the

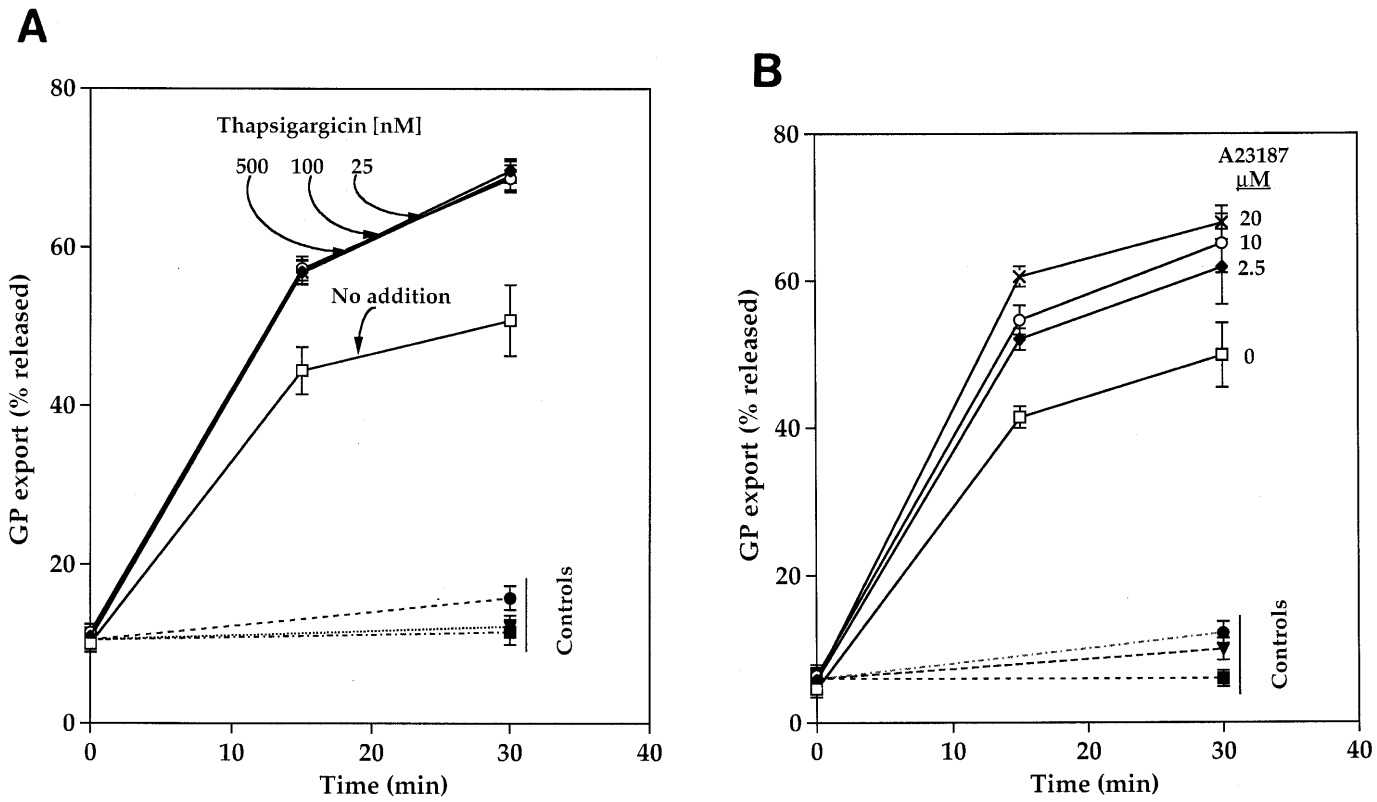


Fig. 2. Free ER lumenal Ca^{2+} is not required for glycopeptide export from rat liver microsomes. (**A**) Time-course of glycopeptide export from rat liver microsomes in the presence of thapsigargicin. The glycopeptide-loaded membranes were incubated at 32°C for the specified period of time in the presence of the following: cytosol only (solid squares), ATP only (solid circles); cytosol and 500 nM thapsigargicin (inverted solid triangles); cytosol and ATP (open squares); cytosol, ATP and 25 nM (diamonds), 100 nM (open circles), 500 nM (×) thapsigargicin. The percentage of glycopeptide released into the media was quantified as described in *Materials and methods*. Each time point is the average of a duplicate determinations ± SE. (**B**) A time-course of glycopeptide release from microsomes in the presence of calcium ionophore A23187. The glycopeptide-loaded membranes were incubated at 32°C for the specified period of time in the presence of the following: cytosol only (solid squares), ATP only (solid circles); cytosol and 20 μM A23187 (inverted solid triangles); cytosol and ATP (open squares); cytosol, ATP and 2.5 μM (diamonds), 10 μM (open circles), 20 μM (×) A23187. The percentage of glycopeptide released into the media was quantified as described in *Materials and methods*. Each data point is the average of two independent determinations ± SE.

control reactions (with ATP or cytosol alone) had no effect indicating that the drug did not interfere with membrane integrity directly, and therefore the effect is specific. Further, we tested the effects of Ionomycin and A23187, calcium ionophores that alter ER lumenal Ca^{2+} concentration by equalizing the concentrations in the lumen and cytosol. These compounds had stimulatory effects in a similar manner to thapsigargicin (Figure 2B and data not shown). Again the presence of these ionophores in the control reactions at relatively high concentrations had no effect indicating specificity. It is clear that both classes of agents, thapsigargicin and calcium ionophores, which interfere with ER lumenal Ca^{2+} concentrations by different mechanisms, are very similar in terms of stimulation of export activity. This is in contrast with the results obtained for oligosaccharides of the polymannose-type export where complete inhibition of glycan transport was observed at the concentrations tested here for glycopeptide export (Moore, 1998). These results indicate that the two processes are probably distinct from one another at least in terms of the importance of Ca^{2+}; oligosaccharide export requiring Ca^{2+} while glycopeptide does not, with ER lumenal free Ca^{2+} being slightly inhibitory to glycopeptide export.

Processing by ER glucosidases is not required for glycopeptide export

We next wanted to determine if the glycan structure and processing of the glycopeptide is a determinant for export efficiency. We approached this by testing the effect of blocking trimming of the α-glucose units that are normally present on oligomannose glycans. It is well documented that the three glucose residues are rapidly removed after transfer of the carbohydrate to the protein by ER glucosidases I and II (Elbein, 1991) and that the transient reglucosylation of the mannose structure is an important feature of ER quality control systems (Helenius *et al.*, 1997). We added either deoxynojirimycin or castanospermine, ER glucosidase inhibitors (Elbein, 1991), prior to loading the membranes with the peptide to block glucose trimming upon addition of the glycan to the peptide and then carried out export of the glycopeptide in the continued presence of the glucosidase inhibitor (Figure 3A for DNJ and not shown for castanospermine which gave similar results). Blocking of glucose trimming had no effect on export of the glucosylated glycopeptide and DNJ-treated membranes exported glycopeptide slightly faster than the untreated microsomes. DNJ had no effect on export of the deglucosylated glycopeptide, as DNJ added during the export reaction,

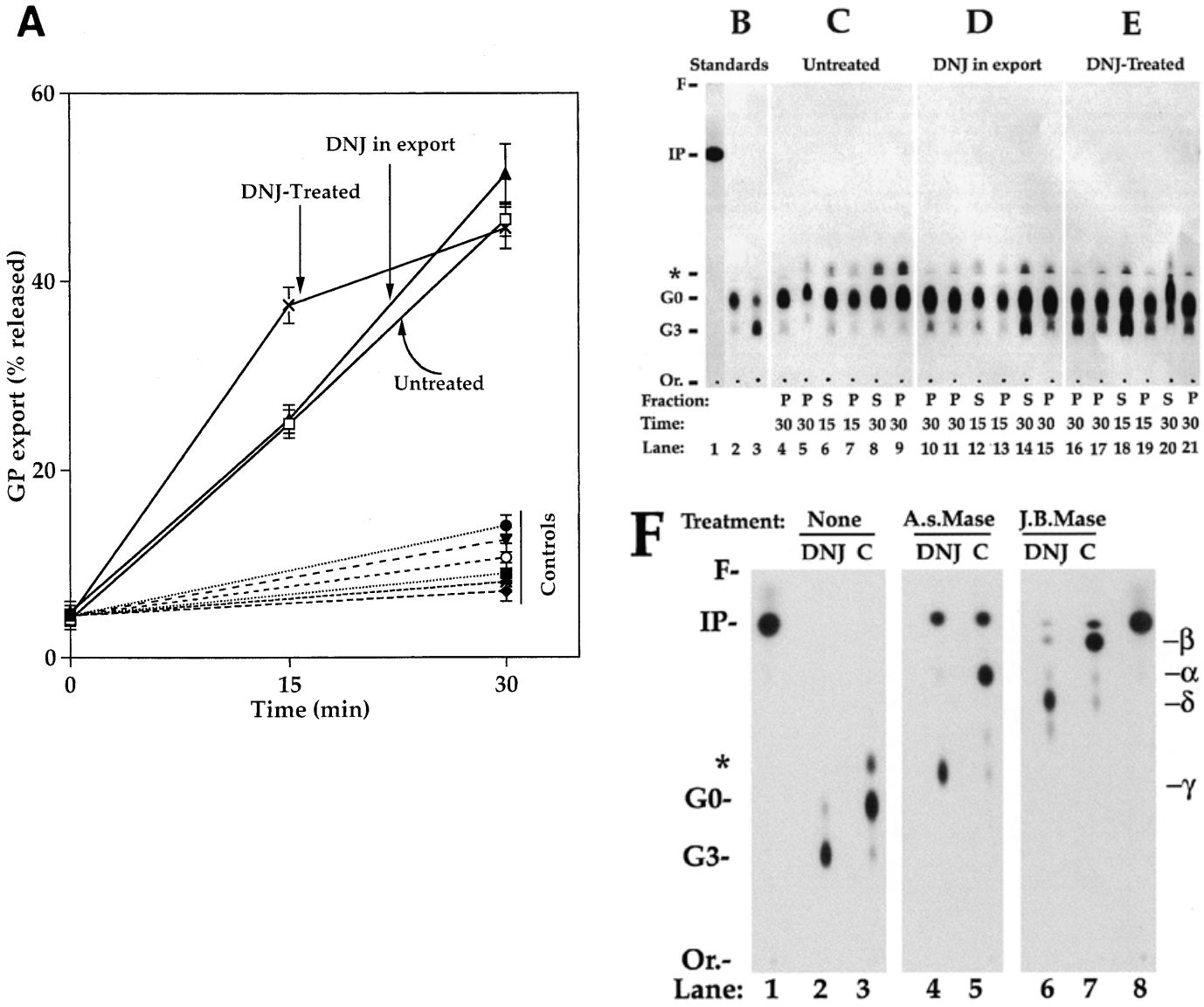


Fig. 3. Effect of ER glucosidase inhibitor DNJ on glycopeptide export. (**A**) Time-course of export reactions in the presence or absence of DNJ. Rat liver microsome aliquot was split after washing step into two portions and kept on ice. To one portion, DNJ was added to 0.5 mM (termed DNJ-treated hereafter) and both tubes were kept on ice for 10 min, to allow the DNJ to inhibit the glucosidase. Iodinated tripeptide was added to both samples and were incubated as described in *Materials and methods*. The DNJ-treated portion was washed with 0.5 ml B88 buffer containing 0.5 mM DNJ and the untreated sample was washed with B88 buffer. The DNJ-treated membranes were used in glycopeptide export reactions in the continued presence of 0.5 mM DNJ as the following: cytosol only (diamonds); ATP only (inverted solid triangles); cytosol and ATP (×). The untreated microsomes were also used for export reactions either without (untreated), cytosol only (solid squares); ATP only (solid circles); cytosol and ATP (open squares) or with DNJ added in the export stage (DNJ in export), cytosol only (asterisks); ATP only (open circles); cytosol and ATP (solid triangles). (**B–E**) Qualitative analysis by TLC of glycopeptides after isolation from the above export reactions. Glycopeptides were isolated as described in *Materials and methods* and ~2000 cpm from selected samples were loaded onto HPTLC silica gel 60 plates and developed as in *Materials and methods* then visualized by autoradiography at –80°C for 48 h. (**B**) Starting material: lane 1, iodinated peptide (Ac-N^{125}IYT-NH_2); lanes 2 and 3, glycopeptides from untreated and DNJ-treated microsomes immediately following loading with glycopeptide, respectively. (**C**) Glycopeptides from export reactions from untreated membranes. Lanes 4 and 5 are from the membrane (P) fraction of cytosol and ATP only reactions due to lack of exported glycopeptides, respectively. Lanes 6–9 are from cytosol and ATP reactions. Incubation time is indicated in the second row from bottom and (P) or (S) designate whether the glycopeptide was isolated from supernatant or pellet of the export reactions, respectively. (**D**) glycopeptides from export reactions from DNJ in export samples. Lanes 10 and 11 are from cytosol and ATP only reactions, respectively, and lanes 12–15 are from cytosol and ATP reactions. (**E**) Glycopeptides from export reactions from DNJ-treated samples. Lanes 16 and 17 are from cytosol and ATP only reactions, respectively, and lanes 18–21 are from cytosol and ATP reactions. The position of standards are indicated on the lefthand side; F, solvent front, IP, iodinated peptide; *, fast migrating glycopeptide (probably fully deglucosylated and ER mannosidase trimmed species); G0, deglucosylated glycopeptide; G3, glucosylated glycopeptide; and Or., origin. All samples were separated on the same TLC plate but split for presentation. (**F**) Glycopeptides from exported material from control (C, untreated) and DNJ-treated (DNJ) samples were purified as described in *Materials and methods* and 30,000 cpm were dissolved in 10 µl of the appropriate buffer containing α-mannosidase (*A.saitoi*, A.s.Mase or jack bean, J.B.Mase) and incubated at 37°C for 24 h; 3000 cpm (1 µl) from each sample were loaded onto TLC plate and chromatographed as in methods. Lanes 1 and 8, iodinated tripeptide. Lanes 2 and 3, undigested glycopeptides from DNJ-treated and control samples, respectively. Lanes 4 and 5, as in 2 and 3 but glycopeptides were digested with *A.saitoi* α-mannosidase. Lanes 6 and 7, as in lanes 2 and 3 but were digested with jack bean α-mannosidase. On the left are markers and on the right are products generated from enzymatic digestions.

allowing deglucosylation to occur, DNJ (in export) was indistinguishable from untreated reactions. DNJ had no effect if added alone or in combination with ATP or cytosol only demonstrating no effects on microsome integrity (see controls in Figure 3A). In addition, quantitation of total ConA recoverable iodinated material from DNJ-treated samples was not significantly less than from untreated samples.

The presence of the glucosylated glycopeptide in the DNJ-treated supernatant fractions was confirmed by the isolation and thin layer chromatography (TLC) analysis of glycopeptides from the same reactions as in Figure 3A. The TLC analysis is shown in Figure 3B–E. Lane 1 shows input peptide, while lanes 2 and 3 show the glycopeptides isolated from control and DNJ-treated membranes after the loading step and before the export step of the assay, respectively. The slower moving glycopeptide in lane 3 is presumably glucosylated glycopeptide (G3) and in lane 2 is the deglucosylated form (G0). Lanes 4–9 are the glycopeptides isolated from the supernatant (S) and membrane (P) fractions of the export reactions from untreated membranes. Glycopeptide from these reactions is predominantly in the deglucosylated (G0) form; there is also a faster moving species of the glycopeptide (*), presumably derived by further processing of the glycopeptide by ER mannosidase I. Glycopeptide from the export reactions performed using DNJ-untreated membranes but with DNJ added during the export step of the assay are similar to the untreated reactions (compare lanes 4–9 with 10–15) with the exception that there is a slower moving (comigrating with G3) glycopeptide, presumed to be a result of glycosylation, during glucosidase inhibitor treatment, of unwashed peptide from the loading step.

There is very little ongoing glycosylation during the export stage of the assay, under standard conditions as described in *Materials and methods*, as evidenced by the constant amount of total glycopeptides recovered (supernatant plus membrane fractions) before and after incubation (not shown). In addition, analysis of total iodinated material, isolated by SepPack from membranes after the washing step and fractionated by TLC, comprises less than 10% unglycosylated peptide (not shown). Ongoing glycosylation in the DNJ-treated samples is due to incomplete washing of membranes as this step was modified to minimize handling time, and which leads to a larger proportion of unwashed tripeptide remaining. Chromatography of glycopeptides derived from export reactions from DNJ-treated membranes prior and during loading and the export steps demonstrate that the glucosylated glycopeptide (G3) is exported out of the ER membranes upon addition of ATP and cytosol (Figure 3B, lanes 16–21). There is some deglucosylation with time even in the continued presence of DNJ, and this may be due to incomplete inhibition of ER glucosidases or processing during subsequent sample handling where the inhibitor concentration may fall below the inhibiting concentration. However, it is clear that fully glucosylated glycopeptides are exported out of the ER and therefore we conclude that processing is not required for export.

To confirm that the slower-moving glycopeptide (G3) in DNJ-treated samples is indeed glucosylated, we isolated glycopeptides from untreated (control) and DNJ-treated samples and performed α-mannosidase digestions on the isolated material (Figure 3F). Untreated material from control reactions migrated essentially as two species, a slower moving major species (G0) and a minor more mobile species (*). On digestion with *A.saitoi* α-mannosidase, both converted to a faster moving species (lane 5, α) consistent with removal of several Manα1–2 residues. The faster moving species (which was also present in lane 4 and comigrating with IP) is probably a result of a contaminated endoH-like activity as it comigrated with endoH digested glycopeptides and was slower than IP using a different solvent system (not shown). Jack bean α-mannosidase digestion of glycopeptide from control reactions resulted in even faster mobility (lane 7, β) than *A.saitoi* mannosidase product which reflects the broader specificity of the jack bean enzyme which removes Manα1–3 in addition to Manα1–2. By contrast, DNJ-treated glycopeptide has lower mobility than control glycopeptide after digestion with either *A.saitoi* (lane 4, γ) or jack bean (lane 6, δ) α-mannosidase. This is consistent with a larger glycan structure on glycopeptide from DNJ-treated samples and indicative of the presence of terminal α-glucose residues which protect the terminal mannose. We were unable to demonstrate sensitivity of glycopeptide from DNJ-treated to digestion with yeast α-glucosidase under several assay conditions; this is likely due to the lower activity of the yeast glucosidase towards high complexity substrates.

Mannosides have weak inhibitory effects on glycopeptide export

From previous work, the precise mannose configuration of polymannose oligosaccharides was demonstrated to have a role in the specificity of oligosaccharides export (Moore, 1998), suggesting involvement of a lectin-like activity in the process. Therefore, we chose to explore the possible role of lectin-like activities in the specificity of glycopeptide export. As the oligosaccharides attached to the glycopeptide are mainly composed of mannose, and in the absence of manipulation, are normally deglucosylated and hence terminate in α mannose, we tested a number of mannose-containing compounds (Figure 4 and Table II). Mannose (M) and methyl-mannose (Me-M) have very little effect on export at 10 mM (Figure 4) but showed about 25% inhibition at 25 mM and stronger inhibition at 100 mM (Table II). Of the dimannoside tested, only Mα[1-6]M showed inhibition, (~20%) at 10 mM, while Mα[1-2]M, Mα[1-3]M, Mα[1-4]M have no effect even at 20 mM. Benzyl-containing mannosides, benzyl-mannose and benzyl-Mα[1-6]M showed about 50% inhibition at 10 and 5 mM concentrations, respectively (Figure 4) and about 60% at 25 and 10 mM (Table II), respectively. From these data, there is an indication that α-1–6 bonding, i.e., Mα1–6M, in a mannobiose unit may influence glycopeptide export but the effect is not strong. At the concentrations tested above, oligosaccharide export was inhibited more potently (Moore, 1998); for example, Mα[1-2]M and Mα[1-3]M showed 50–60% inhibition at 1 mM concentration. Although some mannosides showed some inhibition towards glycopeptide export, the extent and specificity of such inhibition is significantly different from the inhibition of oligosaccharide export, again suggesting that the two processes are distinct from each other.

Discussion

The retrograde translocation from the ER lumen to cytosol of proteins, glycopeptides and free oligosaccharides has recently become a recognized property of the endoplasmic reticulum,

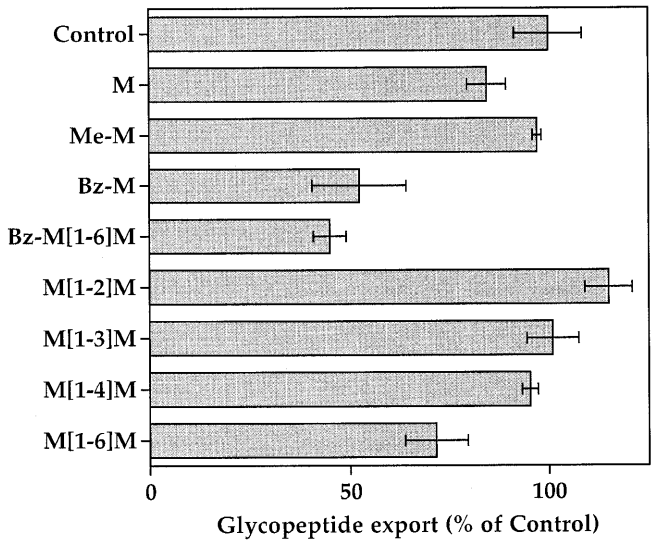


Fig. 4. The effect of mannosides on glycopeptide export from rat liver microsomes. Various mannosides were added to the export step of the assay at 10 mM with the exception of Bz-Mα[1-6]M, (5 mM) and export was performed at 32°C for 20 min. Background export (ATP only) was subtracted from each sample then individual reactions were normalized to the control (cytosol and ATP). All the reactions were performed at the same time using the same microsome aliquot and the values are the average of three independent determinations ± SE. M, mannose; Me, methyl; Bz, benzyl.

Table II. Effect of mannosides on glycopeptide export

Compound	Concentration [mM]	Export (% of control)
None	—	100 ± 5.0
Mannose	25	78 ± 3.8
	100	32 ±4.5
α-Methylmannose	25	72 ± 3.5
	100	44 ± 2.7
Bz-mannose	25	41 ± 5.2
Bz-Mα [1-6]M	10	14 ± 3.2
Mα[1-4]M	20	106 ± 4.2

Mannosides were added to the export stage of the assay and incubated at 32°C for 15 min. Values are the average of two independent determinations ± SE and were normalized to ATP and sytosol (100%) after subtracting the ATP-alone background value. M, Mannose; Bz, benzyl.

and is critically related to the quality control function of the ER (Suzuki *et al.*, 1998). At least some misfolded proteins and glycoproteins are exported via the Sec61p translocon complex indicating the bidirectional nature of movement of macromolecules across the ER membrane (Wiertz *et al.*, 1996; Pilon *et al.*, 1997; Plemper *et al.*, 1997). However, little is known about the mechanisms with which glycopeptides and free oligosaccharides are exported from the ER. Here we address some of the biochemical requirements for glycopeptide export and compare them with free polymannose oligosaccharide export. Both glycopeptide and oligosaccharides require ATP-hydrolysis for export (Romisch and Schekman, 1992; Romisch and Ali, 1997; Moore *et al.*, 1995), and further similarity between the export of the two substrates is the requirement for Mg^{2+} (Moore *et al.*, 1995, and this study), which cannot be substituted by Zn^{2+} or Mn^{2+}. Mg^{2+} dependence may be related to ATP requirement of export or in relation to changes to phospholipid conformation of the ER membrane which make export more efficient.

However, we were able to highlight several major differences between the transport of the two substrates, which leads us to suggest that they are exported, at least partly, by a different mechanisms. First, in oligosaccharide export from HepG2 permeabilized cells (Moore *et al.*, 1995), neither Ac-NYT-NH_2-glycopeptide nor fully glucosylated polymannose oligosaccharides were exported, whereas, the same glycotripeptide is exported with half-time of 8–15 min from both yeast semi-intact cells and rat liver microsomes (Romisch and Schekman, 1992; Romisch and Ali, 1997). Lack of detectable export of glycopeptide from HepG2 permeabilized cells (Moore *et al.*, 1995) might be explained by the lack of cytosolic factors in the export reactions. Additionally, the presence of N-glycanase activity in the cytosolic compartment (Suzuki *et al.*, 1994) or associated with ER membrane may render the exported glycopeptide undetectable due to the immediate hydrolyses to oligosaccharide and peptide moieties upon emergence from the ER (Wiertz *et al.*, 1996). This aspect of glycopeptide export from mammalian microsomes has been recently addressed (van Leyen *et al.*, 1994; Romisch and Ali, 1997).

Most significantly, we found fully glucosylated glycopeptides are exported from rat liver microsomes and therefore the removal of the terminal α-glucose units is not essential for export. This is in contrast to free oligosaccharide export (Moore *et al.*, 1995). Second, glycopeptide, but not free oligosaccharide export requires cytosolic factors in addition to ATP. The glycopeptide cytosolic factor from rat liver cytosol can be fractionated by conventional chromatographic techniques and in fact was resolved from the N-glycanase activity by ammonium sulfate precipitation and ion exchange chromatography (see *Materials and methods*). Third, oligosaccharide export is inhibited by depletion of Ca^{2+} from the assay media or by interfering with free ER lumenal Ca^{2+} using the ER-Ca^{2+}-ATPase specific inhibitor, thapsigargin, and calcium ionophores (Moore, 1998). Glycopeptide export does not require Ca^{2+} in the assay buffer, and was stimulated by ~25% when ER Ca^{2+} mobilizing agents thapsigargicin, A23187 and ionomycin were present. This indicates that a Ca^{2+}-dependent factor partially mediates retention of glycopeptide within the ER lumen. It is also possible that removal of Ca^{2+} may result in some changes to the phospholipid conformation of the ER membrane to make the export more efficient. It is also worth noting that degradation of some, but not all, ER misfolded proteins can be stimulated by altering free ER lumenal Ca^{2+} concentration using thapsigargin (Lodish *et al.*, 1992; Wainwright and Field, 1997). However, it remains to be established if glycopeptide export is related to misfolded protein retranslocation and degradation by the proteasome. Significantly, export of glucosylated substrates has been well documented for glycoproteins (Duvet *et al.*, 1998), but not for free oligosaccharides, consistent with a greater similarity between glycopeptide and glycoprotein retrograde translocation than glycopeptide and oligosaccharide export.

Fourthly, oligosaccharide export was potently inhibited by low millimolar concentrations of mannosides (Moore, 1998) whereas very little or no inhibition was observed when mannosides were added to glycopeptide export systems. For example, Mα[1-2]M and Mα[1-3]M inhibited oligosaccharides by 50–60% at 1 mM (Moore, 1998) but had no effect on glycopeptide export at 10–20 mM. The only significant inhibition observed for glycopeptide export was with benzyl-containing mannosides and with dimannoside Mα[1-6]M. The latter may indicate that α-1,6 mannose linkage is a determinant for export of glycopeptide. Further work will be required to substantiate this hypothesis. The data presented here clearly indicate that glycopeptide and oligosaccharides ER-to-cytosol transport are distinct processes, at least under the *in vitro* conditions used, and further define the glycopeptide export mechanism.

Materials and methods

Chemicals

Acetyl-asparagine-tyrosine-threonine-amide (Ac-NYT-NH_2) was synthesized by ALBACHEM Ltd., UK. ATP; Ionomycin; calcium ionophore A23187; GDP-mannose; concanavalin A Sepharose; mannose; α-methylmannose; benzyl-α-D-mannopyranoside; 2-O-α-D-mannopyranosyl-D-mannopyranoside (2α-mannobiose); 3-O-α-D-mannopyranosyl-D-mannopyranoside;4-O-α-D-mannopyranosyl-D-mannopyranoside; 6-O-α-D-mannopyranosyl-D-mannopyranoside; benzyl 6-O-α-D-mannopyranosyl-α-D-mannopyranoside were all from Sigma Chemical Co. (UK). Castanospermine, Deoxynojirimycin, and Thapsigargicin were from Calbiochem (UK). ^{125}I (100 mCi/ml) was from Amersham-Pharmacia. Rat livers were from Harlan Sera-Lab Ltd. (UK). Creatine kinase and creatine phosphate were from Roche Diagnostics. Other chemicals were obtained from commercial sources and were of the highest purity available.

Iodination of peptide

The peptide (Ac-NYT-NH_2) was iodinated with ^{125}I using chloramine T as described by Wieland *et al.* (1987). The iodinated peptide was purified from unincorporated ^{125}I by binding to Sep-Pack C_{18} (Waters Chromatography PLC) light cartridge then elution with 60% acetonitrile in 0.1% TFA. Iodinated peptide was used within 1 month of iodination.

Preparation of rat liver membranes and partially purified rat liver cytosol

Rat liver rough membranes and cytosol were prepared as described previously (Romisch and Ali, 1997). Cytosol was prepared from frozen rat livers purchased from Harlan Sera-Lab Ltd. (Loughborough, UK) and rough microsomes from fresh livers obtained from the departmental facility. The cytosol was fractionated to remove N-glycanase activity (Suzuki *et al.*, 1994) which compromises detection of the exported glycopeptide (Romisch and Ali, 1997). The glycopeptide export activity was precipitated by a 40–60% ammonium sulfate cut. The precipitated proteins were dissolved in 20 mM Tris–HCl, pH 8.0, containing 1 mM DTT then dialyzed against the same buffer. The proteins were then loaded onto a Q-Sepharose fast flow column preequilibrated in the same buffer and eluted with a 0–1.0 M KCl gradient. The glycopeptide export active fractions eluted between 0.3 and 0.5 M KCl and were free of N-glycanase activity. Active fractions were pooled and dialyzed against 20 mM Tris–HCl, pH 7.5, containing 0.5 mM DTT and stored in small aliquots at –80°C until use.

Glycopeptide export assay from rat liver microsomes

The glycopeptide export assay was performed as described in Romisch and Ali (1997) with some modifications in the following three steps.

Loading. Crude rat liver membranes (100 µl, A_{280} ~200) were suspended in 1 ml of ice-cold B88 buffer (20 mM HEPES-KOH, pH 7.4, 150 mM KOAc, 250 mM sorbitol and 5 mM $Mg[OAc]_2$) containing 0.5 M KCl or NaCl and placed on a rotator for 15 min at 4°C. The membranes were then sedimented in a cooled (4°C) microfuge (Eppendorf) at 20,000 × *g* for 5 min and then washed with 1 ml of B88 buffer (pH 7.4). The membranes were then resuspended in B88 buffer (200 µl) and ^{125}I-Ac-NYT-NH_2 was added at 1×10^7 c.p.m./100 µl and the reaction mix incubated at 10°C for 20 min. This allows the peptide to enter the ER membranes and become glycosylated by the endogenous oligosaccharyltransferase. To remove unglycosylated tripeptide, 2 × 1 ml of ice-cold B88 buffer was added and the membranes were then sedimented as above and resuspended in 200 µl of B88 buffer.

Export. Loaded membranes (5 µl of washed membranes, ~A_{280} = 10) were placed in Eppendorf tubes on a pre-cooled ice-cold block and the various components, as necessary, were added to individual tubes in the following order: B88 buffer to bring the final volume to 25 µl, 2.5 µl partially purified rat liver cytosol (5 µg protein), inhibitor or other compound and 2.5 µl of a 10X ATP-regenerating system (final concentrations; 1 mM ATP, 40 mM creatine phosphate, 0.2 mg/ml creatine kinase and 50 µM GDP-mannose). Export reactions were initiated by incubating the tubes at 32°C for a specified period of time (typically 30 min) after which the membranes were quickly sedimented at 4°C in microfuge and the supernatant (cytosol) separated from the pellet (membranes).

Quantitation of glycopeptide export. To each supernatant or pellet fraction, 100 µl of 2% SDS was added and samples were heated immediately at 95°C for 5 min. The samples were cooled, and 1 ml of ConA buffer (20 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 1% Triton X-100) was added followed by 50 µl of 50% immobilized ConA Sepharose slurry and the mix placed on a rotator at room temperature for 2 h. The beads were then sedimented using a microfuge and washed once (by suspending then sedimenting by brief centrifugation) with each of the following: IP buffer (15 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS), urea buffer (100 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 2 M urea) and finally with ConA buffer. Radioactivity was then measured by γ-counting using an LKB 1275 minigamma counter. Glycopeptide export was determined as the percentage of radioactivity (cpm) in supernatant relative to the total counts in supernatant and membranes.

Isolation of glycopeptides, digestion with α-exoglycanases and thin layer chromatography

Glycopeptides were purified exactly as described (Romisch and Ali, 1997). Thin layer chromatography (TLC) analysis was performed using Merck HPTLC aluminum backed silica gel 60 plates and developed in *n*-propanol:acetone:water (9:5:6 (v/v)) in a saturating atmosphere and visualized by autoradiography at –80°C. Digestions with yeast α-glucosidase (Roche Diagnostics), jack bean (Sigma) and *A.saitoi* (Oxford Glycosciences) α-mannosidases were performed on 30,000 cpm glycopeptides in 10 μl of buffer according to manufacturer's recommendations at 37°C for 24 h. One microliter from each reaction was loaded onto a TLC plate and chromatographed as above.

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Abbreviations

TLC, thin layer chromatography; DTT, dithiothreitol; cpm, counts per minute; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; ERAD, endoplasmic reticulum associated degradation; ER, endoplasmic reticulum; DNJ, deoxynojirimycin; ConA, concanavalin A; HEPES, 4-(2-hydroxyethyl)-1-piperirazinethansulfonic acid; SE, standard error; M, mannose; Me-M, α-methylmannose; Bz-M, Benzyl-α-D-mannopyranoside; Mα[1-2]M, 2-O-α-D-mannopyranosyl-D-mannopyranoside; Mα[1-3]M, 3-O-α-D-mannopyranosyl-D-mannopyranoside; Mα[1-4]M, 4-O-α-D-mannopyranosyl-D-mannopyranoside; Mα[1-6]M, 6-O-α-D-mannopyranosyl-D-mannopyranoside; Bz-Mα[1-6]M, Benzyl 6-O-α-D-mannopyranosyl-α-D-mannopyranoside.

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