

Similar processes mediate glycopeptide export from the endoplasmic reticulum in mammalian cells and *Saccharomyces cerevisiae*

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ABSTRACT Glycopeptides are transported from the lumen of the yeast endoplasmic reticulum (ER) to the cytosol and in contrast to secretory proteins do not enter ER-to-Golgi transport vesicles. In a cell-free system, this process is ATP- and cytosol-dependent. While yeast cytosol promotes the export of glycopeptides from mammalian ER *in vitro*, glycopeptide release cannot be detected in the presence of mammalian cytosol. We demonstrate that this is due to an N-glycanase activity in mammalian cytosol rather than lack of glycopeptide transport activity in mammalian microsomes. Monitoring the amount of glycopeptide enclosed in ER membranes we show the cytosol- and ATP-dependent release of glycopeptide from mammalian microsomes. The fact that glycopeptide export can be achieved with ER and cytosol derived from heterologous sources indicates that glycopeptide export from the ER is an important process conserved during evolution.

In eukaryotic cells the endoplasmic reticulum (ER) plays a key role in the quality control of secretory and membrane proteins (1). Only if signal peptide cleavage, core-glycosylation, folding, and oligomerization are completed successfully, are secretory proteins packaged into ER-to-Golgi transport vesicles (1). Misfolded or incompletely oligomerized proteins are retained in the ER and often degraded (1). So far, very little is known about the mechanism of recognition for ER degradation, but recent evidence suggests that at least some misfolded secretory proteins are not degraded in the lumen of the ER, but in the cytosol (2, 3). As summarized below, several different lines of research suggest that smaller molecules, such as glycopeptides, oligopeptides, and free polymannose oligosaccharides, also do not remain within the secretory pathway, but are actively transported from the ER lumen to the cytosol.

Glycopeptides are exported from the yeast ER by a mechanism that is distinct from the secretory pathway (4). In a yeast-based *in vitro* system acceptor peptides for oligosaccharyl transferase enter the ER and are core glycosylated, but in contrast to secretory proteins they are not packaged into ER-to-Golgi transport vesicles (4). Instead, they are directly transported across the ER membrane to the cytosol in an ATP and cytosol-dependent manner (4). These glycopeptides do not acquire Golgi-specific α 1,6-linked mannose (4) and no significant amount of glycopeptide is secreted by intact spheroplasts (K.R., unpublished work).

ER-to-cytosol transport of oligopeptides in mammalian cell-free systems has been observed in several laboratories (5, 6). In mammalian cells, the transporter associated with antigen presentation imports a variety of peptides derived from "foreign," e.g., viral, proteins from the cytosol into the ER lumen (7). Because

the transporter associated with antigen presentation is more promiscuous than the major histocompatibility class I complex, not all imported peptides can bind to the major histocompatibility class I complex (7). Free peptides are transported back to the cytosol in an ATP- and temperature-dependent fashion (5, 6).

Moore and colleagues (8, 9) demonstrated free polymannose oligosaccharide export from mammalian ER to the cytosol in intact and in permeabilized cells. *In vitro*, this process was ATP- and temperature dependent (9). The transported oligosaccharides consisted of eight or nine mannose residues and one or two *N*-acetyl-D-glucosamine (GlcNAc) residues (8). The authors suggested that the di-*N*-acetyl chitobiose oligosaccharides were generated by hydrolysis of core oligosaccharides off dolichol in the absence of acceptor secretory proteins (8, 9). Oligosaccharides carrying a single GlcNAc residue were derived from glycoproteins and possibly end products of ER degradation (8). As free oligosaccharides are substrates for processing enzymes in the Golgi, Moore and Spiro (8) postulated that removal of free oligosaccharides from the secretory pathway was required to prevent competition with the processing of secretory glycoproteins.

Given the evidence described above, it seemed likely that mammalian ER was able to export glycopeptides, yet van Leyen and colleagues (10) observed no glycopeptide export from mammalian microsomes. The possibility remained, however, that glycopeptides were transported across the ER membrane, but subsequently modified by mammalian cytosol and therefore escaped detection. Here we demonstrate that in the presence of yeast cytosol dog pancreas microsomes can indeed export glycotriptides. Mammalian cytosol also promotes glycopeptide export, but a cytosolic N-glycanase specific for mammalian cytosol removes the glycan moiety and thus renders the peptide undetectable by lectin affinity chromatography. The fact that glycopeptide transport can be achieved with ER and cytosol from heterologous sources suggests that the proteins involved in this process are functionally conserved between yeast and mammals.

MATERIALS AND METHODS

Strains, Growth Conditions, and Preparation of Cell Fractions. The *Saccharomyces cerevisiae* strains used were RSY255 (*MAT α* , *ura3-52*, *leu2-3*, *112*) and RSY607 (*MAT α* , *ura3-52*, *leu2-3*, *112*, *PEP4::URA3*). Yeast cells were grown to early logarithmic phase at 30°C in yeast extract/peptone (2% Bacto-peptone and 1% yeast extract, both from Difco) containing 2% or 5% glucose. ³⁵S-labeled prepro- α -factor was synthesized *in vitro* as described (4). Yeast cytosol (RSY607) and semi-intact cells (RSY255) were prepared as described (4). For rat liver cytosol and microsomes adult rats were starved for 24 hr, killed, and the livers homogenized in B88 (20 mM Hepes/150 mM potassium acetate/250 mM sorbitol/5 mM magnesium

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Abbreviations: ER, endoplasmic reticulum; endo H, endoglycosidase H; GlcNAc, *N*-acetyl-D-glucosamine.

acetate, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF)/1 mM DTT at 4°C. The homogenate was cleared of large debris by 10 min centrifugation at 8,000 × g. The supernatant was subjected to centrifugation for 40 min at 100,000 × g. The supernatant was gel-filtered through a Bio-Rad G10 column in B88 containing 1 mM PMSF/1 mM DTT. The protein-containing fractions were pooled and frozen in 100-μl aliquots in liquid nitrogen and stored at -80°C. Protein concentration was 35 mg/ml. The membrane pellet from the 100,000 × g spin was washed in B88 (pH 7.4), containing 1 mM PMSF/1 mM DTT once, and frozen in B88 (pH 7.4) in 100-μl aliquots at an A₂₈₀ (in 1% SDS) of 25 (crude rat microsomes). Crude rough microsomes from dog pancreas were gifts from Peter Walter (University of California, San Francisco) and Stephen High (University of Manchester) and prepared as described (11). *N*-acetyl-NYT-NH₂ was synthesized by David King (University of California, Berkeley) and iodinated using chloramine T as described (12). Specific activity was 2–3 × 10⁷ cpm/nmol.

Yeast Semi-Intact Cell-Based Glycopeptide Export Assay. This assay is done as described by Römisch and Schekman (4).

Glycopeptide Export from Dog Pancreas and Rat Microsomes. Dog pancreas microsomes (A₂₈₀ = 30) were diluted 1:5 in B88 (pH 7.4), containing 1 × 10⁷ cpm/100 μl of ¹²⁵I-Ac-NYT-NH₂, 1 mM ATP (Sigma), 40 mM creatine phosphate (Boehringer Mannheim), 0.2 mg/ml creatine phosphokinase (Boehringer Mannheim), 50 μM GDP mannose (Sigma), and incubated at 10°C for 15 min. Membranes were washed twice in B88 (pH 7.4), and 2.5 μl microsomes (A₂₈₀ = 30) were incubated with ATP and cytosol in 25 μl total volume as indicated in the specific experiment. At the end of the incubation, samples were chilled on ice for 5 min, and membranes sedimented for 4 min at 4°C in a microfuge. Supernatant and pellet were heated to 95°C for 5 min in 1% SDS and glyco-

peptide in each fraction precipitated with concanavalin A (Con A)-Sepharose (Pharmacia). After thawing, crude rat microsomes were incubated with 0.5 M KCl in B88 (pH 7.4) for 15 min at 4°C to remove membrane-associated cytosolic factors. The membranes were sedimented and washed once in B88 (pH 7.4) prior to incubation with the acceptor peptide. We used 3.75 μl of washed rat microsomes at A₂₈₀ = 25 per 25 μl export reaction.

Reverse-Phase Analysis of Peptides. The supernatant fractions from yeast export reactions were incubated with endoglycosidase H (endo H) or PNGase F, or mock incubated, then heated to 95°C in the presence of 1% SDS, diluted in B88, and loaded onto SepPak C₁₈ light cartridges (Waters/Millipore). The cartridges were washed with 1 ml of water and eluted in 1 ml steps with 2%, 5%, 10%, 20%, and 50% acetonitrile. Iodinated peptide in each fraction was detected by γ counting. Digestions with endo H (Boehringer Mannheim) or PNGase F (Boehringer Mannheim) were performed at 0.1 unit/ml and 20 units/ml, respectively, for 1 hr at 37°C.

Purification of Peptides and Thin-Layer Chromatography. Glycopeptides were isolated from the supernatant fractions of yeast export reactions by binding to Con A-Sepharose (Pharmacia) and elution with 0.5 M α-methyl D-mannoside. The eluate was subsequently passed over a SepPak C₁₈ light cartridge, and peptides eluted with 60% acetonitrile. Peptides were dried down in aliquots and reconstituted at 9 × 10⁴ cpm/10 μl in either water, water containing 0.1 unit/ml endo H, 20 units/ml PNGase F, or 7 mg/ml mammalian cytosol. Reactions were incubated for 1 hr at 37°C. About 1 × 10⁴ cpm per lane were loaded onto a silica plate (HPTLC aluminum sheets, silica gel 60; Merck) and developed in butanol/acetic acid/water at 5:2:2. Chromatograms were analyzed by autoradiography.

pH Dependence of Mammalian Cytosolic N-Glycanase. About 2.2 × 10⁴ cpm of purified glycopeptide was incubated in 50-μl reactions containing 52.5 μg rat liver cytosol and 10 mM of the appropriate buffer. Reactions were incubated for 20 min at 32°C, heated to 90°C in the presence of 2% SDS for 5

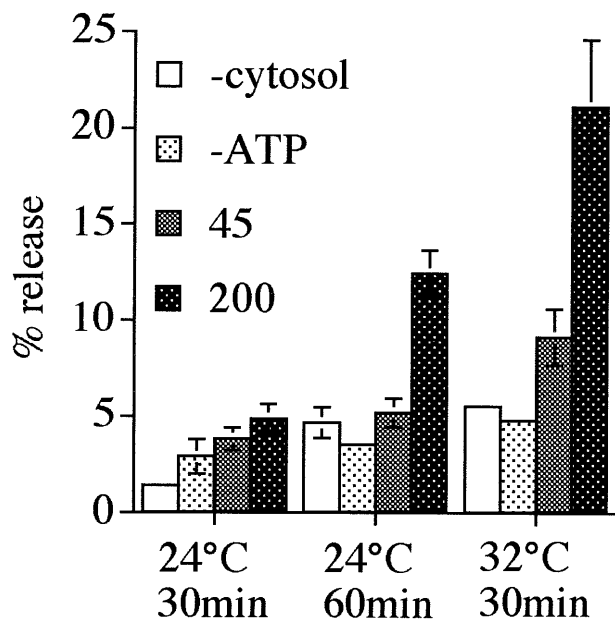


FIG. 1. Yeast cytosol promotes glycotriptide export from dog pancreas microsomes. Aliquots (2.5 μl) of dog pancreas microsomes (A₂₈₀ = 30) were incubated with or without ATP and an ATP-regenerating system, and with or without 45 or 200 μg of yeast cytosol derived from RSY255 in 25 μl for the indicated times at 24°C or 32°C. Samples without cytosol contained ATP and the regenerating system, samples without ATP contained 200 μg of yeast cytosol. Membranes were sedimented and glycotriptide in the supernatant and pellet fractions were quantified by Con A precipitation. All samples were done in duplicate. Glycotriptide released from the membranes at time zero was subtracted as background from each value. Total glycotriptide in each sample was ≈100,000 cpm.

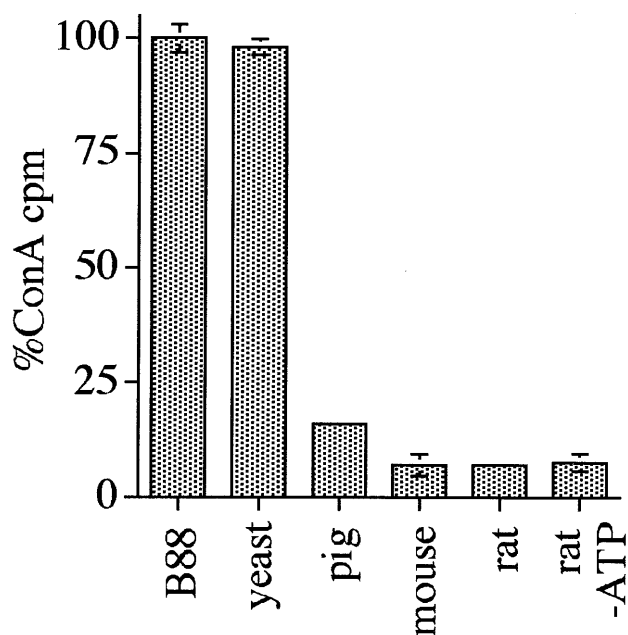


FIG. 2. Mammalian cytosol degrades glycotriptides. Aliquots of glycopeptide (8,200 cpm each) derived from a yeast export reaction were incubated in the presence of B88, 7.3 mg/ml yeast cytosol (RSY607), 13.2 mg/ml pig brain cytosol, 6.7 mg/ml mouse cytosol, or 7.8 mg/ml rat liver cytosol in 45-μl reactions with or without ATP and the regenerating system for 1 hr at 37°C. Reactions were done in duplicate. Glycopeptide was quantified by Con A precipitation.

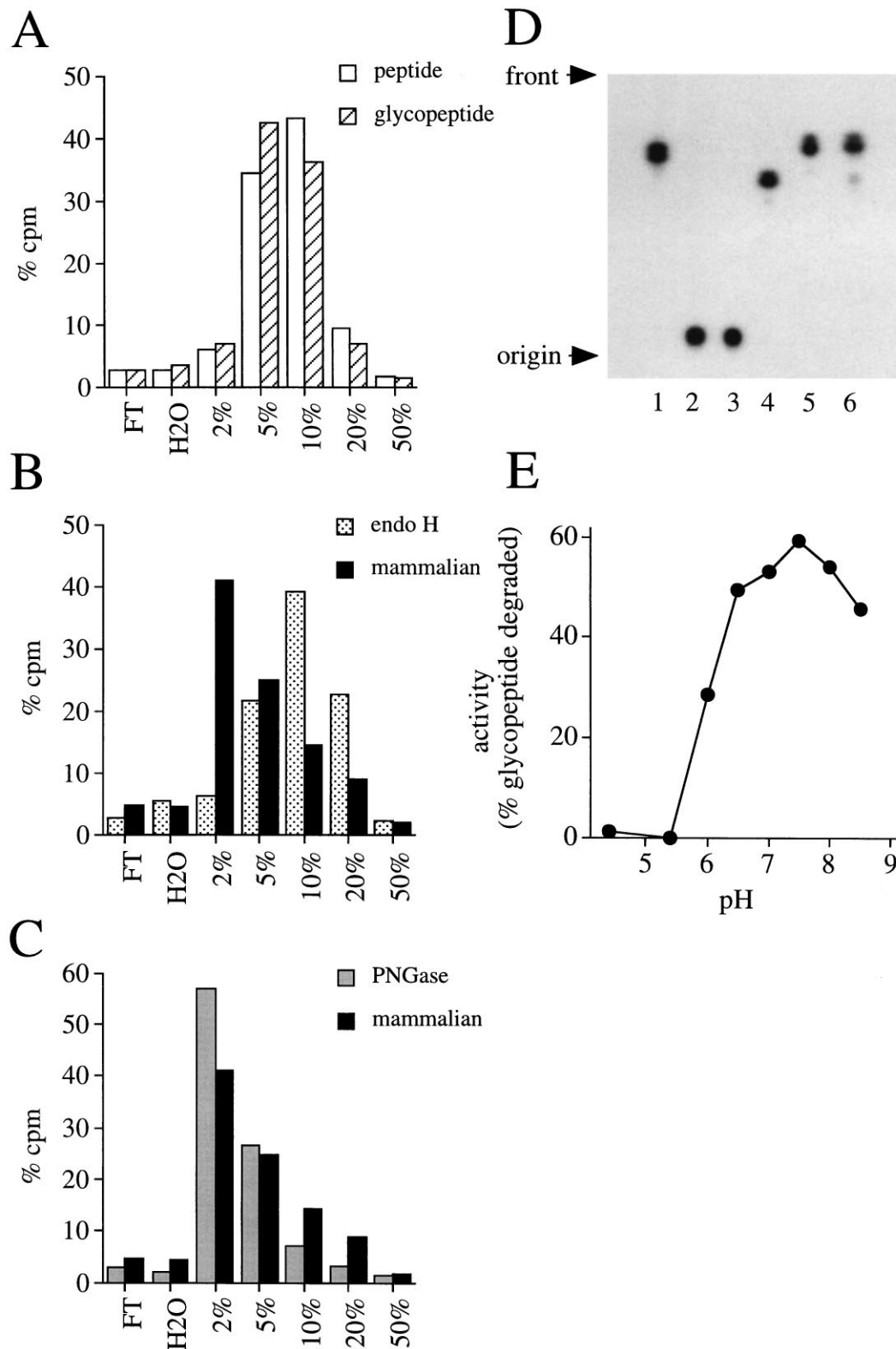


FIG. 3. Mammalian cytosol contains an N-glycanase. (A) Aliquots of $\approx 4 \times 10^4$ cpm of unglycosylated tripeptide or 1.6×10^4 cpm of glycotripeptide derived from a yeast export reaction were loaded onto SepPak C_{18} reverse-phase cartridges and eluted in 1-ml steps with increasing concentrations of acetonitrile as indicated. FT, flowthrough. ^{125}I cpm in each fraction were detected by γ counting. (B and C) Glycotripeptide (1.6×10^4 cpm) derived from a yeast export reaction were incubated with endo H (0.1 unit/ml), PNGase F (20 units/ml), or mammalian cytosol (10 mg/ml) for 1 hr at 37°C and subsequently analyzed by reverse-phase chromatography as above. (D) Purified glycotripeptide was incubated with water (lane 3), endo H (0.1 unit/ml, lane 4), PNGase F (20 units/ml, lane 5), or mammalian cytosol (7 mg/ml, lane 6) for 1 hr at 37°C and analyzed by thin-layer chromatography as described. Lane 1, unglycosylated peptide; lane 2, purified glycopeptide. (E) Aliquots of purified glycotripeptide were incubated with rat liver cytosol for 20 min at 32°C in various buffers as described in *Material and Methods*. At the end of the incubation, remaining glycotripeptide was quantified by Con A-Sepharose precipitation and γ counting, and activity expressed as percent glycopeptide degraded during the incubation.

min, and the remaining glycopeptide was detected by precipitation with Con A-Sepharose and γ counting. Buffers: potassium acetate, pH 4.4; Mes-KOH, pH 5.4; Mes-KOH, pH 6.0; HEPES-KOH, pH 6.5; HEPES-KOH, pH 7.0; HEPES-KOH, pH 7.5; HEPES-KOH, pH 8.0; Tris-HCl, pH 8.5.

RESULTS AND DISCUSSION

Glycosylated Acceptor Tripeptide Is Exported from Mammalian ER in the Presence of Yeast Cytosol and ATP. Transport of a glycotriptide from the yeast ER to the cytosol has been demonstrated previously in a well-characterized cell-free system (4). The export of glycotriptide in this system is dependent on ATP and cytosol (4). In contrast, in a cell-free assay containing mammalian microsomes and mammalian cytosol, glycotriptide could only be found in the lumen of the microsomes (ref. 10; K.R., data not shown). We asked whether glycotriptide could be exported from the mammalian ER in the presence of yeast cytosol. Fig. 1 shows that in the presence of a high concentration of yeast cytosol and ATP (solid bars) glycotriptide is indeed exported from dog pancreas microsomes. In the yeast cell-free system the optimal temperature for export is 24°C, and after 30 min export is close to maximal (20–25% for yeast microsomes; ref. 4). As shown in Fig. 1, export from dog pancreas microsomes was more efficient at 32°C than at 24°C and reached 21.2% after 30 min. An assay temperature of 32°C was chosen for all subsequent experiments because it allowed for the efficient combination of yeast and mammalian components, whereas the export activity in the yeast cell-free system was reduced at 37°C, the physiological temperature for mammalian cells.

Under the experimental conditions used for the experiment in Fig. 1 the secretory protein glyco-pro- α -factor cotranslationally translocated into dog pancreas microsomes was not released from the membranes. Furthermore, under conditions that do not allow glycotriptide export from the ER, both glyco-pro- α -factor and the glycotriptide in the microsome fraction were protected from digestion with endo H, which shows that the membranes were intact. Finally, yeast cytosol preparations that were inactive in promoting glycopeptide export from yeast ER likewise failed to promote glycopeptide export from dog pancreas microsomes. We conclude that in the presence of yeast cytosol, mammalian ER actively exports glycotriptides to the cytosol.

Glycotriptides Are Degraded in Mammalian Cytosol. As shown in Fig. 1, mammalian ER is intrinsically capable of exporting glycotriptides. It therefore seemed likely that export also took place in the presence of mammalian cytosol, but that mammalian cytosol in contrast to yeast cytosol contained an activity that modified the glycotriptide and rendered it undetectable by lectin precipitation. To test this possibility, we incubated radiolabeled glycotriptide with buffer or cytosol from yeast or various mammalian sources, and subsequently precipitated glycotriptide with the mannose-reactive lectin Con A as in our standard export assay. Yeast cytosol or buffer had no influence on the stability of the glycotriptide (Fig. 2), but in all mammalian cytosols tested the glycotriptide was degraded, and no longer bound to Con A (Fig. 2). Degradation was independent of the presence of ATP (Fig. 2). We conclude that glycopeptide degradation is the reason for the inability to detect glycotriptide export from the mammalian ER in the presence of mammalian cytosol (10).

Mammalian Cytosol Contains an N-Glycanase Activity. Both cytosolic β -hexosaminidase and cytosolic N-glycanase activities have been reported in mammalian cells (13–15). β -hexosaminidases cleave the core-oligosaccharyl chain between the two GlcNAc residues, leaving one GlcNAc attached to the acceptor asparagine, and thus do not affect the overall charge of the deglycosylated oligopeptide (16). In contrast, N-glycanases are amidases that convert the aspara-

gine at the site of hydrolysis to aspartate, introducing a negative charge (16). To investigate the nature of the deglycosylating activity in our assay, we analyzed the elution profiles of glycopeptides and deglycosylated peptides from reverse-phase cartridges. Fig. 3A shows that both the acceptor peptide (N-acetyl-NYT-NH₂) and the core-glycosylated form of the same peptide eluted from a C₁₈ reverse-phase matrix at 5% and 10% acetonitrile. Deglycosylation of the glycotriptide with the commercially available β -hexosaminidase endo H resulted in a slight shift toward the hydrophobic end of the elution profile, such that the peptide peak eluted at 10% acetonitrile (Fig. 3B, stippled bars). In contrast, incubation of the glycotriptide with mammalian cytosol caused maximum elution of peptide already at 2% acetonitrile (Fig. 3B and C, solid bars). This elution profile overlapped entirely with the elution profile of peptide deglycosylated with commercially available N-glycanase (Fig. 3C, solid bars vs. shaded bars). In addition, we digested purified glycopeptides with endo H, N-glycanase, or mammalian cytosol, and analyzed the digestion products by thin-layer chromatography. The products derived from N-glycanase and the activity in mammalian cytosol migrated to the same position (Fig. 3D, lanes 5 and 6), whereas the peptide derived from endo H digestion migrated more slowly (Fig. 3D, lane 4). We conclude that a cytosolic N-glycanase is responsible for glycotriptide degradation in cell-free reactions containing mammalian cytosol. As shown in Fig. 3E, this activity has a neutral pH optimum, as expected for a cytosolic enzyme; thus, lysosomal contamination of our cytosol preparations can be ruled out. The N-glycanase may be identical to the activity observed by Wiertz and colleagues (15), which is responsible for the cytosolic deglycosylation of major histocompatibility complex class I heavy chains destined for deg-

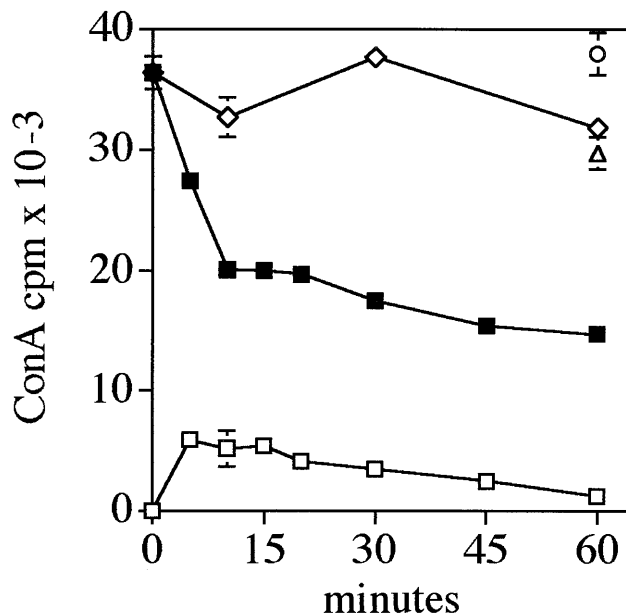


FIG. 4. Mammalian cytosol promotes glycotriptide export from mammalian ER. Salt-washed rat microsomes were loaded with glycotriptide as described. Release reactions were performed in 25- μ l aliquots containing 3.75 μ l microsomes ($A_{280} = 25$), 70 μ g rat cytosol or buffer, and ATP and an ATP-regenerating system. Reactions were incubated at 32°C or 0°C, then stopped at the indicated time points, membranes and supernatant fractions separated by centrifugation, and glycotriptide was quantified in the membrane fractions and in the supernatant fraction by ConA precipitation. All reactions were performed in duplicate. (■), 32°C membranes incubated with ATP and cytosol; (□), 32°C supernatants of above; (◇), 0°C membranes incubated with ATP and cytosol; (△), 32°C membranes, no cytosol; (○), 32°C membranes, no ATP.

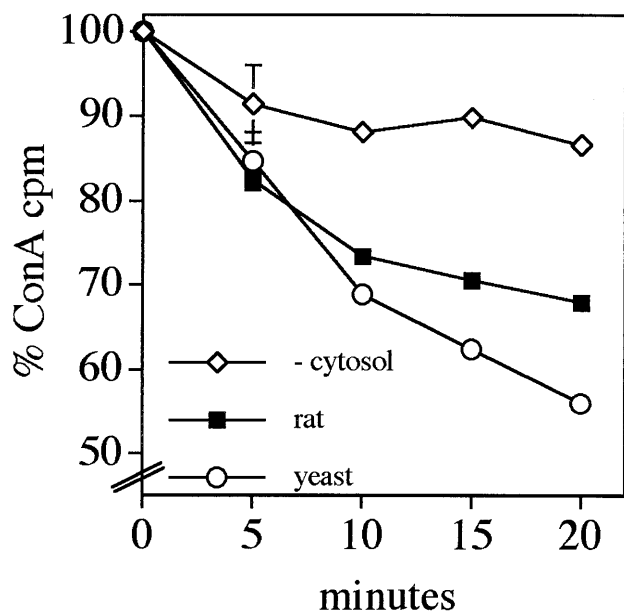


FIG. 5. Mammalian cytosol promotes glycotriptide exit from yeast ER. Export reactions from yeast semi-intact cells were performed at 32°C in the absence of cytosol (\diamond), 350 μ g rat cytosol (\blacksquare), or 75 μ g yeast cytosol (\circ), and ATP- and an ATP-regenerating system in 25- μ l aliquots. At the indicated times, reactions were stopped and glycotriptide in the membrane fractions quantitated by Con A precipitations. Samples were done in duplicate.

radation by the proteasome in the cytosol of cytomegalovirus-infected cells.

Mammalian Cytosol Promotes Glycotriptide Export from Mammalian ER. There are currently no inhibitors of N-glycanase activity available; thus, we decided to monitor glycopeptide export from the ER in the presence of mammalian cytosol by measuring the ATP- and cytosol-dependent decrease of the amount of glycopeptide in the ER lumen. Fig. 4 shows a time course of glycotriptide release from salt-washed rat liver microsomes. Up to 50% of the glycotriptide was exported from the rat microsomes in the presence of ATP and a saturating amount of rat liver cytosol during the incubation time (Fig. 4, solid squares). Note that there was no proportional appearance of glycotriptide in the supernatant fraction due to the N-glycanase activity in mammalian cytosol (Fig. 4, open squares). The $t_{1/2}$ for glycotriptide release was about 8 min (Fig. 4, solid squares), similar to the efficient ATP-dependent export of oligopeptides from mouse microsomes observed by Shepherd and colleagues (17).

Mammalian Cytosol Promotes Glycotriptide Export from Yeast ER. As we initially demonstrated with glycotriptide release from dog pancreas microsomes in the presence of yeast cytosol, it seemed likely that inversely glycotriptide export from the yeast ER could be stimulated with rat cytosol. As shown in Fig. 5, high concentrations of rat cytosol indeed promoted glycotriptide export up to 25% from the ER of semi-intact yeast cells (solid squares). For comparison, glycotriptide release in the presence of a saturating amount of yeast cytosol is shown (Fig. 5, open circles). We conclude that the proteins involved in glycopeptide export from the ER are sufficiently conserved between yeast and mammals to be at least partially functionally interchangeable.

We have compared the behavior of the glycosylation acceptor tripeptide N-acetyl-NYT-NH₂ in yeast and mammalian cell-free reactions containing ER membranes and cytosol. We found that the glycosylated acceptor tripeptide is efficiently exported from the lumen of mammalian ER to the cytosol, similar to the export process that we had described earlier in yeast (4).

Our discovery of an N-glycanase activity in all mammalian cytosols tested explains why glycopeptide export from the mammalian ER has escaped detection previously (10). We cannot exclude, however, that a very small fraction of glycopeptide in the ER lumen is packaged into ER to Golgi transport vesicles and ultimately secreted from mammalian cells as reported (12). No glycopeptide secretion, however, was observed from intact yeast spheroplasts (K.R., unpublished data) or *Xenopus* oocytes microinjected with oligosaccharyl transferase acceptor peptides (18). In the oocytes, the peptides enter the ER and are core-glycosylated but subsequently remain intracellular, which is indicative of their removal from the secretory pathway (18). We conclude that glycopeptide export from the ER to the cytosol is a process that occurs universally in eukaryotic cells.

Like glycotriptide export in yeast, export from the mammalian ER is dependent on ATP and cytosol (Fig. 4). For crude rat liver microsomes, the cytosol-dependence of glycopeptide export was only evident if the membranes were salt-washed prior to use in the export reaction. Thus, it is possible that the solely ATP-dependent oligopeptide export observed from mammalian microsomes by Ploegh and Hämmerling and colleagues (5, 6, 17) is likewise dependent on ER-associated cytosolic factors and mediated by the same machinery that is responsible for glycopeptide export. Furthermore, this process may be related to the export of free oligosaccharides from the ER to the cytosol observed by Moore and colleagues (8, 9) and is possibly representative for the reexport of misfolded secretory glycoproteins from the ER to the cytosol for degradation (2).

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