

The protein translocation channel mediates glycopeptide export across the endoplasmic reticulum membrane

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Peptides and misfolded secretory proteins are transported efficiently from the endoplasmic reticulum (ER) lumen to the cytosol, where the proteins are degraded by proteasomes. Protein export depends on Sec61p, the ribosome-binding core component of the protein translocation channel in the ER membrane. We found that prebinding of ribosomes abolished export of a glycopeptide from yeast microsomes. Deletion of *SSH1*, which encodes a ribosome-binding Sec61p homologue in the ER, had no effect on glycopeptide export. A collection of cold-sensitive *sec61* mutants displayed a variety of phenotypes: two mutants strongly defective in misfolded protein export from the ER, *sec61-32* and *sec61-41*, displayed only minor peptide export defects. Glycopeptide export was severely impaired, however, in several *sec61* mutants that were only marginally defective in misfolded protein export. In addition, a mutation in *SEC63* strongly reduced peptide export from the ER. ER-luminal ATP was required for both misfolded protein and glycopeptide export. We conclude that the protein translocation channel in the ER membrane mediates glycopeptide transport across the ER membrane.

High concentrations of peptides are generated in the endoplasmic reticulum (ER) by several processes: signal peptides are removed from translocating secretory proteins and proteolytically cleaved by a signal peptide-processing enzyme (1), and the ER also contains at least one protease involved in misfolded membrane protein degradation that may generate peptides in the ER lumen (2, 3). In addition, the transporters associated with antigen processing (TAP) efficiently import antigenic peptides from the cytosol into the ER of mammalian cells (4); only a fraction of these peptides can bind to the MHC class I complex and are presented at the cell surface. The vast majority of TAP substrates are rapidly exported from the ER to the cytosol and either are processed further and reimported into the ER or are degraded (5, 6). Removal of suboptimal antigenic peptides from the ER is important for successful MHC class I-mediated antigen presentation. Flavivirus infection of mammalian cells, for example, causes a general increase in peptide concentration in the ER and thus leads to increased presentation of cellular instead of viral antigens at the cell surface (7). In addition, removal of peptides from the secretory pathway at an early stage is essential to prevent competition with secretory proteins for covalent modifications in the ER and Golgi apparatus; nonspecific peptides in the secretory pathway would also compete with binding of peptides derived from extracellular antigens to the MHC class II complex in a late compartment of the secretory pathway (8, 9). Finally, uncontrolled release of nonspecific peptides into the extracellular space would interfere with intercellular communication mediated by neuropeptides or peptide pheromones (10).

Peptide transport from the ER to the cytosol was first discovered by using a synthetic acceptor peptide for oligosaccharyl transferase in a yeast-based cell free assay system designed to study ER-to-Golgi transport (11). Hydrophobic acceptor peptides enter microsomes by an undefined route, possibly by partitioning into the lipid bilayer, and release on the ER-luminal

side where they are core glycosylated. In contrast to secretory proteins, however, glycopeptides are not packaged into ER-to-Golgi transport vesicles; instead, they are transported directly across the ER membrane to the cytosol in an ATP- and cytosol-dependent fashion (11). Most TAP substrates and free polymannose oligosaccharides are also efficiently transported from the ER lumen to the cytosol in an ATP-dependent fashion (5, 6, 8). Initially, retrograde transport across the ER membrane was assumed to be restricted to small molecules and was viewed as a disposal pathway for end products of "ER degradation" of misfolded secretory proteins (11). Recently it became clear, however, that cytosolic proteasomes are responsible for this degradation process, and that misfolded proteins themselves are exported across the ER membrane to the cytosol before degradation (for review, see ref. 12).

At present, the transporters for peptides and oligosaccharides from the ER are still unknown, but the exit pathway for misfolded secretory proteins is well characterized: these proteins are exported from the ER to the cytosol via a channel containing Sec61p, the central component of the protein-conducting channel also responsible for secretory protein import into the ER (13–15). This channel in the ER membrane of both yeast and mammalian cells is formed by the heterotrimeric Sec61 complex, which consists of Sec61p (Sec61 α in mammals), Sbh1p (Sec61 β), and Sss1p (Sec61 γ). Sec61p is an essential polytopic protein with 10 transmembrane domains that line the protein-conducting channel in the ER membrane (16). A nonessential homologue of Sec61p, Ssh1p, forms a complex homologous to the Sec61 complex containing the Sbh1p homologue Sbh2p and Sss1p (17); the Ssh1 complex may have a specialized role in cotranslational translocation into the yeast ER, but Ssh1p is not required for misfolded protein export from the ER (15, 17). During post-translational protein import into the yeast ER, the Sec63 complex interacts with the Sec61 complex, forming the heptameric Sec complex (18, 19). The Sec63 complex consists of four proteins, Sec63p, Sec62p, Sec71p, and Sec72p, and is required for signal-sequence recognition during import into the ER (20). With the possible exception of Sec63p itself, none of the components of the Sec63 complex are required for misfolded protein export from the ER (14, 15).

Recently, we have characterized protein import and export defects in a collection of cold-sensitive *sec61* mutants in yeast *in vivo* and in cell-free assay systems based on yeast microsomes and cytosol; these reproduce protein import and export for degra-

Abbreviations: ER, endoplasmic reticulum; TAP, transporters associated with antigen processing; PDI, protein disulfide isomerase; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid.

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dation of a mutant form of the secretory pheromone precursor prepro- α -factor (Δ gpaf) (14, 21, 22). Here, we have used these cell-free assay systems in conjunction with mutants that are defective in genes important for bidirectional protein transport across the ER membrane to ask whether the protein translocon was the export route of oligopeptides from the ER. We demonstrate that the central translocon component, Sec61p, is indeed required for peptide export from the ER, but also that cells distinguish between peptides and unfolded proteins: screening a collection of *sec61* mutants, we found that specific *sec61* alleles are defective in glycopeptide export from the yeast ER. In addition, ER-luminal ATP and Sec63p are required for glycopeptide export from the ER. Deletion of *DER1* which interferes with misfolded protein export from the ER and mutations in protein disulfide isomerase (PDI) that interfere with its binding to glycopeptides and export of some proteins from the ER had no effect on glycopeptide export. We conclude that glycopeptides, like misfolded proteins, are exported from the ER via channels formed by Sec61p, but that substrate-specific accessory proteins are required for either targeting to or opening of the channel.

Materials and Methods

Strains and Growth Conditions. Strains: RSY155 (*MAT α* leu2-3,-112 ura3-52 ade2-1 pep4-3 sec63-1; ref. 23), RSY255 (*MAT α* leu2-3,-112 ura3-52; ref. 24), RSY524 (*MAT α* leu2-3,-112 ura3-52 ade2-1 pep4-4 sec61-2; ref. 18), RSY529 (*MAT α* leu2-3,-112 ura3-52 his4-619 sec62; ref. 23), RSY1132 (*MAT α* leu2-3,-112 ura3-52 trp1-1 sec61-3; ref. 24), RSY1293 (*MAT α* can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura3-1ade2-1 sec61::HIS3 pDQ1[*sec61-his6*] or pDQ1 expressing the indicated *sec61* mutants; ref. 22), KRY139 (*MAT α* can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura3-1ade2-1sec61::HIS3 pAC8[*SEC61-SUC2*]; ref. 16), KRY140 (as KRY139 but *SEC61*), WCG4a (*MAT α* leu2-3,-112 ura3 his3-11,-15; ref. 15), YTX87 (*MAT α* can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 ade2-1; ref. 17), YTX84 (as YTX87 but Δ ssh1::LEU2; ref. 17), JLY1 (*MAT α* can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura3-1ade2-1 pdi1::HIS3 pRS314 [*PDI1*]; 25), JLY551 (as JLY1 but Δ 252-277pdi1; ref. 25), JLY550 (as JLY1 but Δ 222-302pdi1; ref. 25), CTY182 (*MAT α* ura3-52 Δ his3-200 lys2-801; ref. 26), CTY244 (as CYT182 but *sac1-296::HIS3*; ref. 26). Yeast were grown in YPD [1% yeast extract/2% peptone (Difco)/2% dextrose] or synthetic dextrose media with the appropriate additions (27).

Cell Fractionation. Cells were grown at 24°C (RSY155, RSY529, RSY524, RSY1132) or 30°C (all others) and microsomes prepared as described (14). Cytosol for glycopeptide export was prepared by bead beating from RSY255 (11). Cytosol for misfolded protein export and degradation was prepared from WCG4a by liquid nitrogen lysis. Ribosomes were prepared by centrifugation from liquid nitrogen lysed yeast cytosol (28); the supernatant was used as ribosome-free cytosol, and the ribosomal pellet was taken up in an equal volume of B88 (20 mM Hepes, pH 6.8/150 mM potassium acetate/250 mM sorbitol/5 mM magnesium acetate)/1 mM DTT.

Peptide Iodination. Glycosylation acceptor tripeptide (Ac-NYT-NH₂) was synthesized commercially and iodinated by using chloramine T (11). Specific activity was at least 2×10^7 cpm/nmol.

Glycopeptide Export from Yeast Microsomes. As described for dog pancreas microsomes (29): yeast microsomes ($A_{280} = 30$) were diluted 1:5 in B88, pH 6.8, containing 2×10^7 cpm/100 μ l of ¹²⁵I-Ac-NYT-NH₂, 1 mM ATP (Sigma), 40 mM creatine phosphate (Boehringer Mannheim), 0.2 mg/ml creatine phosphokinase (Boehringer Mannheim), 50 μ M GDP-mannose (Sigma),

incubated at 10°C for 15 min, then washed in B88, pH 6.8, and 2- μ l microsomes ($A_{280} = 30$) were incubated with ATP and 50 μ g cytosol in 25 μ l total volume as indicated. At the end of the incubation, samples were chilled on ice for 2 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 glycopeptide in each fraction precipitated with Con A-Sepharose (Pharmacia) and quantified by γ -counting. Individual samples contained $0.5\text{--}2 \times 10^4$ Con A-precipitable cpm; variations were caused by variations in oligosaccharyl-transferase activity in *sec61* mutants and reflect membrane protein integration defects (22), not variations in peptide uptake into mutant microsomes. Within this range, glycopeptide concentration did not affect export from wild-type microsomes, and acceptor peptide did not significantly inhibit glycosylation of secretory proteins. The ratio of glycosylated ¹²⁵I-acceptor tripeptide in the supernatant to total glycosylated ¹²⁵I-acceptor tripeptide in the reaction (supernatant plus pellet) is expressed as percent release. Microsomes were not pretreated for inhibition of glycopeptide export by ribosomes, and inhibition was alleviated by dissociating ribosomes with EDTA (10 mM) or digestion with RNase A (100 μ g/ml, 10 min, room temperature). For inhibition of glycopeptide export by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), the indicated concentrations were included in the wash buffer at the end of the translocation reaction and in the export reaction.

ER Degradation Assay. ER degradation of the nonglycosylated form of pro- α -factor (Δ gpaf) (30) was assayed as in ref. 21. For inhibition of Δ gpaf export by DIDS, the indicated concentrations were included in the wash buffer at the end of the translocation reaction and in the export reaction.

Results

Ribosomes Inhibit Glycopeptide Export from the ER. Misfolded secretory proteins are transported from the ER lumen to the cytosol via a channel containing Sec61p, the principal component of the protein translocation channel in the ER membrane responsible for secretory protein import into the ER lumen (13–15). Ribosomes bind specifically to Sec61p in the ER membrane (31), and Johnson and colleagues have demonstrated that flux of ions through the protein translocation channel in the ER membrane can be inhibited by prebinding of nontranslating ribosomes to Sec61p in microsomal membranes (32). We asked whether incubation of yeast microsomes with ribosomes had any effect on glycopeptide export. We prepared microsomes from wild-type yeast and imported a ¹²⁵I-labeled glycosylation acceptor peptide, *N*-acetyl-Asn-Tyr-Thr-amide, into the lumen of these microsomes, where the peptide was core glycosylated. After washing, the membranes were incubated in the presence of nontranslating ribosomes in buffer, buffered cytosol depleted of ribosomes, or buffer alone for 10 min at 24°C. We observed no unspecific glycopeptide release during the preincubation, indicating an intact membrane barrier. We then added ATP, an ATP-regenerating system, and yeast cytosol, and incubated reactions for 30 min at 24°C. Supernatant and membrane fractions were separated by centrifugation and glycopeptide in each fraction quantitated by lectin precipitation with Con A-Sepharose followed by γ -counting. As shown in Fig. 1, preincubation with ribosomes, but not with ribosome-free cytosol, completely blocked glycopeptide export from the ER. Our data suggest that a channel in the ER membrane that can be blocked by binding of ribosomes is responsible for glycopeptide export.

Ssh1p Is not Required for Glycopeptide Export from the ER. The ER contains at least two channels that bind ribosomes: one is the protein translocon in the ER membrane whose principal component is Sec61p; the other is a similar channel whose principal component is a nonessential Sec61p homologue, Ssh1p (17). We prepared microsomes from a Δ ssh1 strain and the isogenic wild

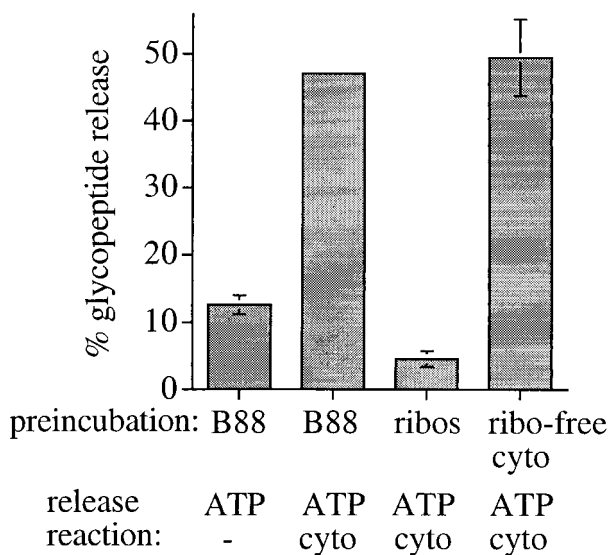


Fig. 1. Ribosomes inhibit glycopeptide export from the ER. Yeast cytosol (18 mg/ml) was separated by centrifugation into ribosomes and a ribosome-free supernatant fraction and the ribosomal pellet resuspended in the original volume B88. Wild-type microsomes [RSY255, 2 μ l microsomes ($A_{280} = 30$) per sample] were translocated with 125 I-Ac-NYT-NH₂, washed, and incubated for 10 min at 24°C in 12.5 μ l B88, 12.5 μ l B88 containing nontranslating ribosomes, or 12.5 μ l ribosome-free cytosol, as indicated. Glycopeptide export was assayed subsequently for 30 min at 24°C in the absence (–cytosol) or presence (all others) of cytosol and ATP, as described in *Methods*. Samples contained $2\text{--}5 \times 10^4$ Con A precipitable cpm, were done in duplicate, and the experiment was repeated three times.

type and monitored glycopeptide export from these membranes in the presence of ATP and cytosol over time as described above. As shown in Fig. 2, Δ *ssh1* microsomes were fully competent for glycopeptide export. In good agreement with Plemper and colleagues, who found that Ssh1p is not required for export of a mutant form of carboxypeptidase Y (CPY*) from the ER *in vivo*, we found that Δ *ssh1* microsomes displayed no defects in Δ gpaf export from the ER *in vitro* (ref. 15 and data not shown). We

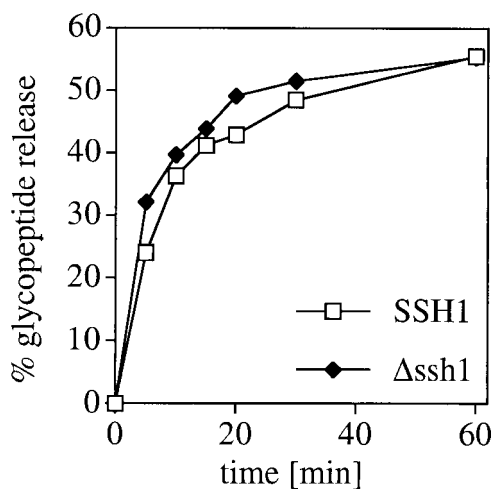


Fig. 2. Ssh1p is not required for glycopeptide export. Wild-type (*SSH1*) and Δ *ssh1* microsomes were assayed for glycopeptide export as described in *Methods*. At individual time points, duplicate samples were transferred to ice, the membranes sedimented by centrifugation, and glycopeptide in the supernatant fraction analyzed by Con A precipitation and γ -counting.

conclude that Ssh1p is involved in neither glycopeptide nor misfolded protein export from the ER.

Specific Mutants in *sec61* Are Defective in Glycopeptide Export from the ER to the Cytosol. A collection of new mutants in *sec61* that are cold sensitive for protein import into the ER are also defective to various degrees in misfolded protein export to the cytosol, with *sec61-32* and *sec61-41* displaying the strongest defects (14, 22). These mutants encode stable Sec61p (22). We prepared microsomes from strains grown at the permissive temperature (30°C) and characterized them in the cell-free glycopeptide export assay. We first investigated two mutants, *sec61-32* and *sec61-41*, strongly defective in misfolded protein export from the ER (Fig. 3 *Lower*; refs. 14 and 22). To our surprise, these mutants were only moderately affected in their capacity to export glycopeptides (Fig. 3 *Upper*; 30% export for *SEC61* (wild type); 26% export for *sec61-32*; 23% export for *sec61-41*). In other *sec61* mutants (*sec61-10*, *sec61-11*, *sec61-23*, *sec61-24*), however, there was a strong correlation in the defects in protein import, misfolded protein export, and glycopeptide export (Fig. 3; refs. 14 and 22). Furthermore, overexpression of wild-type *SEC63* in *sec61-10* and *sec61-24* strains, which partially alleviates their cold sensitivity and protein translocation defects, also suppressed the glycopeptide export defect in these mutants (not shown). All mutants contain an amino-terminal 6-histidine-tag that on its own has no effect on any of the processes investigated (ref. 22 and Fig. 3; compare *SEC61* and *sec61-his6*). In combination with additional point mutations in *sec61*, however, the His-tag contributes to the mutant phenotype, as illustrated by the partial rescue of the glycopeptide export defect in *sec61-110*, which is identical to *sec61-10* but has its His-tag removed (Fig. 3). Similarly, removal of the His-tag from *sec61-10* resulted in a substantial recovery of both protein import into and misfolded protein export from the ER in this strain (Fig. 3 *Lower*; ref. 22).

Several of the *sec61* mutants displayed differential defects for protein translocation (import or export) across the ER membrane and glycopeptide export from the ER: *sec61-32*, *sec61-41*, and *sec61-7* microsomes were strongly defective in protein translocation across the ER membrane (Fig. 3 *Lower*; ref. 22; M.P., unpublished work) but were affected only moderately in glycopeptide export (Fig. 3 *Upper*). By contrast, *sec61-2*, a previously isolated temperature-sensitive mutant, displayed only moderate defects in protein transport across the ER membrane (Fig. 3 *Lower*; ref. 22) but was strongly deficient in glycopeptide export (Fig. 3 *Upper*). Microsomes from *sec61-8* cells displayed the strongest of all observed glycopeptide export phenotypes (Fig. 3 *Upper*) but were only mildly defective in misfolded protein export from the ER (Fig. 3 *Lower*) and had an intermediate phenotype for protein import (M.P., unpublished work). Note that *sec61-3* microsomes were fragile and released glycopeptide in the absence of cytosol; the effect of *sec61-3* on glycopeptide transport across the ER membrane could therefore not be evaluated. All other *sec61* mutant microsomes were stable (Fig. 3 *Upper*). Microsomes from a strain expressing a *SEC61-SUC2* fusion in which the carboxyl-terminal 21 amino acids of Sec61p were replaced by invertase as its sole source of Sec61p were affected only marginally in protein transport across the ER membrane in either direction but showed a 50% reduction in glycopeptide export compared with wild type (ref. 16 and data not shown). Our data suggest that glycopeptide transport from the ER lumen to the cytosol requires Sec61p and that distinct structural features of the Sec61 channel are important for glycopeptide and misfolded protein export.

Glycopeptide Export from the ER Depends on Sec63p. The differential effects of a subset of our *sec61* mutant alleles on glycopeptide export and misfolded protein export from the ER indicate that, whereas the pathway for both substrates across the membrane is

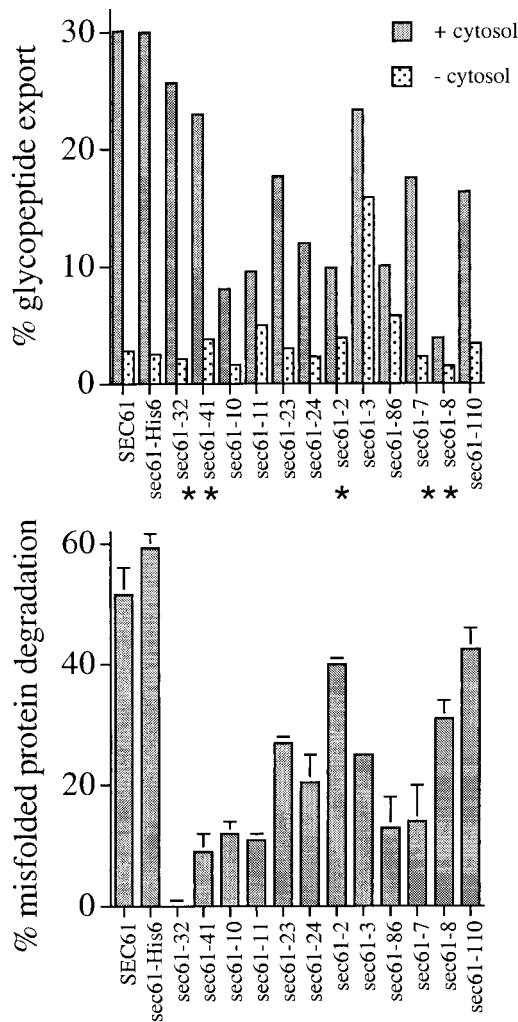


Fig. 3. Specific *sec61* mutants are defective in glycopeptide export from the ER. Microsomes were prepared from *SEC61* wild-type and mutant strains grown at their respective permissive temperatures (see *Methods*). (Upper) Glycopeptide export. Each strain was assayed in duplicate for glycopeptide export for 30 min at 24°C in the presence or individual absence of ATP and cytosol, as described in *Methods*. Release at 30 min is shown. Nonspecific release in the absence of ATP was less than 5% and subtracted. Mutants with significantly different effects on glycopeptide and misfolded protein export are marked with asterisks. (Lower) Misfolded protein export. Mutant α -factor precursor ($p\Delta gpa\alpha$) was translocated into wild type or *sec61* mutant microsomes and $\Delta gpa\alpha$ export and degradation initiated by the addition of ATP and cytosol, as described in *Methods*. After 30 min at 24°C, proteins were precipitated with TCA, resolved by gel electrophoresis, and $\Delta gpa\alpha$ quantified by using a Bio-Rad phosphorimager. Samples were done in duplicate and the experiment repeated twice.

identical, either the mechanism of channel opening or the accessory molecules involved may be different. We therefore investigated the impact of mutations in accessory molecules involved in protein transport across the ER membrane on glycopeptide export. First, we examined the effects of temperature-sensitive mutations in the genes encoding Sec62p and Sec63p, two transmembrane ER proteins that are subunits of the translocon during posttranslational protein import into the ER (18, 23). Although both proteins are essential for import, Sec62p is not required for misfolded protein export from the ER, and a mutant allele of *SEC63*, *sec63-1*, causes only a 2- to 3-fold increase in half life of misfolded proteins in the ER (14, 15).

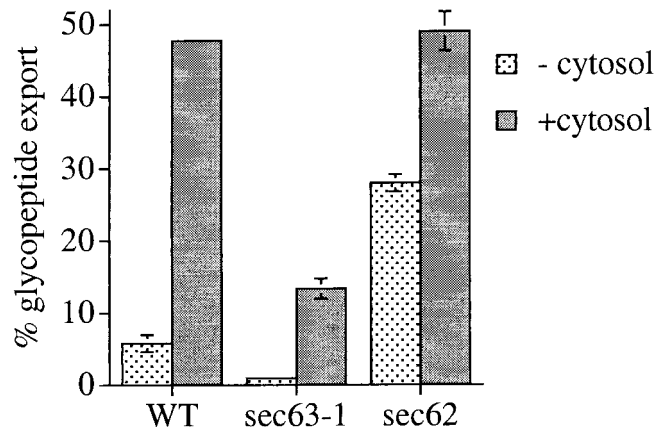


Fig. 4. Glycopeptide export from the ER depends on Sec63p. Microsomes were prepared from wild-type and *sec62* or *sec63* mutant cells grown at the permissive temperature (see *Methods*) and glycopeptide export assayed as described in Fig. 1.

Mutant *sec62* microsomes were structurally defective like *sec61-3* membranes and released a significant fraction of glycopeptide in the absence of cytosol (Fig. 4). We were therefore unable to evaluate the impact of this mutation on glycopeptide transport through the Sec61 channel. Membranes derived from *sec63-1* mutant cells, however, were strongly defective in glycopeptide export from the ER compared with wild type (Fig. 4; 48% export wild type; 13% export *sec63-1*), suggesting that Sec63p is essential for glycopeptide export from the ER.

A screen for mutants defective in ER-associated degradation of a mutant vacuolar protease, CPY*, by Wolf and colleagues, resulted in the identification of several genes encoding polytopic ER membrane proteins (33). We analyzed the mutant with the strongest defect in CPY* degradation, $\Delta der1$, for export and degradation of another mutant secretory protein precursor, $\Delta gpa\alpha$, and for glycopeptide export (33). We found that the half life of $\Delta gpa\alpha$ in *der1* ER was moderately increased in both intact cells and in a cell-free assay for ER-associated degradation (2-fold by pulse-chase, 3-fold *in vitro*). By contrast, deletion of *DER1* had no effect on glycopeptide export from the ER (data not shown). Taken together, our data suggest that different subsets of ER membrane proteins cooperate with the Sec61 channel during export of misfolded proteins and glycopeptides from the ER.

Release from PDI Is Not Critical for Glycopeptide Export from the ER Lumen.

The ER-luminal enzyme and chaperone PDI is the predominant binding partner in the ER lumen for glycosylation acceptor peptides and unglycosylated peptides irrespective of their thiol content (34–36). PDI is composed of four thioredoxin modules, two enzymatically active ones (a, a') and two inactive ones (b, b') (37). Klappa and colleagues (37) found that the PDI b' domain is sufficient for binding of peptides, but that other domains of PDI contribute to unfolded protein binding and thus to the chaperone function of the protein. We have shown that deletion of 25 amino acids of the yeast PDI b' domain results in decreased glycosylation acceptor peptide binding (Pdi $\Delta 252$ –277; 40% peptide binding of wild type; ref. 25), and that deletion of the amino-terminal half of the b' domain and a small proportion of the preceding b domain reduces peptide binding to PDI to background levels (Pdi $\Delta 222$ –302; ref. 25). To investigate the effects of these deletions on glycopeptide export from the ER, we prepared microsomes from wild-type and *pdi1* mutant strains and measured glycopeptide export over time for 90 min in the presence of ATP, an ATP-regenerating system, and

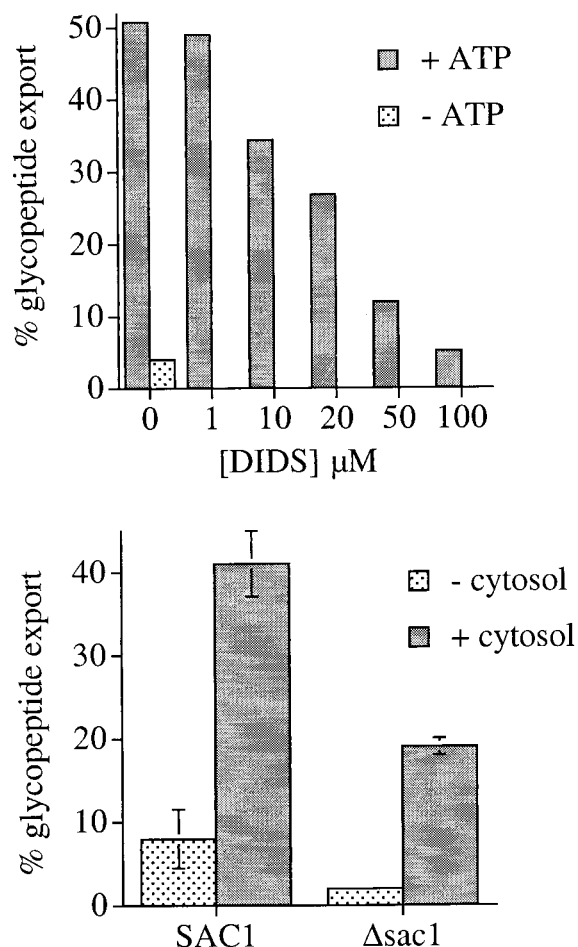


Fig. 5. Glycopeptide export across the ER membrane requires ATP in the ER lumen. (Upper) DIDS inhibits glycopeptide export from the ER. Wild-type (RSY255) microsomes were translocated with ^{125}I -Ac-NYT-NH $_2$, washed with B88 containing the indicated concentration of DIDS, and glycopeptide export in the presence of the indicated concentrations of DIDS assayed in duplicate as described in Fig. 1. (Lower) Δsac1 microsomes are defective in glycopeptide export from the ER. Microsomes were prepared from wild-type (SAC1) and Δsac1 cells and glycopeptide export assayed as described in Fig. 1.

wild-type yeast cytosol. We found no difference in the kinetics and the degree of glycopeptide export from wild-type and mutant strains, suggesting that release from PDI is not limiting for glycopeptide export from the ER (data not shown).

Misfolded Secretory Protein Export and Glycopeptide Export Across the ER Membrane Are *Sac1p* Dependent and Can Be Inhibited by DIDS.

Both misfolded secretory protein export and glycopeptide export from the ER depend on ATP, but it remains unclear which specific steps are ATP dependent (11, 21). We asked whether ATP was required in the ER lumen. We performed peptide export experiments in the presence of the anion transport inhibitor DIDS, a stilbene derivative that inhibits anion transporters and interferes with transport of ATP into the ER lumen (30). As illustrated in Fig. 5 Upper, we found that DIDS inhibits glycopeptide export from microsomes in a dose-dependent fashion. Export was maximally inhibited in the presence of 100 μM DIDS (Fig. 5 Upper). Misfolded secretory protein export from the ER *in vitro* was similarly inhibited by DIDS (not shown).

Sac1p is a multifunctional nonessential phosphoinositide phosphatase in the ER membrane that regulates ATP transport

into the ER lumen (38, 39). Deletion of *SAC1* results in reduced protein translocation into the ER (40). We investigated the effect of Δsac1 on glycopeptide export from the ER and found glycopeptide export from Δsac1 microsomes was reduced by 50% compared with wild type (Fig. 5 Lower). Deletion of *SAC1* had a similar effect on misfolded secretory protein export from the ER (not shown). In summary, our data suggest that ATP is required in the ER lumen for both glycopeptide and misfolded secretory protein export.

Discussion

Our results demonstrate that the central translocon component, Sec61p, is required for glycopeptide export from the ER. This is an important function of the Sec61 channel in addition to its well-established role as a protein-conducting channel. Glycopeptide flux through the Sec61 channel could be blocked by binding of non-translating ribosomes to its cytosolic face. By screening a collection of *sec61* mutants in a cell-free assay system, we found that specific *sec61* alleles were defective in glycopeptide export from the yeast ER. In addition, ER-luminal ATP and Sec63p were required for glycopeptide export from the ER. The lack of correlation between defects in protein import and glycopeptide export in several of our *sec61* mutants suggests that the peptide export defects were not an indirect consequence of reduced membrane protein integration, but rather that a channel formed by Sec61p was directly involved in glycopeptide export from the ER.

Sec61p is a transmembrane protein of 480 amino acids with 10 transmembrane domains and both termini exposed to the cytoplasm (16). Addition of a 6 His-tag to the amino terminus of Sec61p on its own had no effect on any of the Sec61p functions tested (Fig. 3, *SEC61* vs. *sec61-His6*). Nevertheless, the tag contributed to the mutant phenotypes in combination with other point mutations in *SEC61*, suggesting that modification of the amino terminus destabilizes Sec61p structure. The location of the mutations in the cold-sensitive *sec61* mutant alleles used in this study has been established (22): four alleles (*sec61-32*, *sec61-41*, *sec61-86*, *sec61-24*) have single amino acid substitutions in transmembrane domains III and IV of Sec61p. The notion that this region is structurally important for channel formation is supported by the fact that *sec61-24* (L162P) and *sec61-86* (G140D) affected misfolded protein and glycopeptide export to a similar degree (Fig. 3). By contrast, *sec61-32* (C150Y) and *sec61-41* (V134I) primarily interfered with protein transport through the Sec61 channel, suggesting a specific functional defect. Pilon *et al.* (22) have shown that *sec61-32* is defective in channel insertion of secretory proteins during import into the ER. In misfolded protein export assays, the substrate proteins remained associated with the luminal face of the Sec61 channel in *sec61-32* and *sec61-41* mutants, suggesting again a defect in polypeptide chain insertion into the channel in these mutants (14). The fact that glycopeptide export can proceed from *sec61-32* and *sec61-41* ER indicates distinct mechanisms of polypeptide and glycopeptide entry into the Sec61 channel from the ER lumen.

All other cold-sensitive *sec61* mutant alleles harbor multiple amino acid changes (22). Those affecting peptide export are clustered in transmembrane domains III and IV, the carboxyl-terminal transmembrane domain, and the carboxyl-terminal cytoplasmic tail of Sec61p. Replacement of the carboxyl-terminal 21 amino acids of Sec61p with a bulky molecule of invertase also led to a significant reduction in glycopeptide but not misfolded protein export from the ER, which may indicate that this region of Sec61p is important for interaction with the cytosolic factor required for glycopeptide export from the ER. The identity of this factor remains unknown, but it is different from the cytosol requirements for misfolded protein export from the ER (K.R., unpublished work).

Binding of ribosomes to Sec61 channels almost certainly occludes the cytosolic carboxy terminus of Sec61p and may sterically hinder binding of the cytosolic factor required for export. Preincubation of ER membranes with ribosomes had no significant effect on misfolded secretory protein export from the ER *in vitro* (Fig. 1; K.R., unpublished work), suggesting that in contrast to glycopeptide exit, misfolded protein exit from the ER may be initiated from the lumen such that it cannot be inhibited by ribosome binding to the channel on the cytoplasmic face. Alternatively, glycopeptides and misfolded proteins may be transported from the ER via two different populations of Sec61 channels; this notion is supported by our observation that glycopeptide and misfolded protein export do not compete with each other (K.R., unpublished data). Creation of stable export intermediates that block the Sec61 channel may ultimately help resolve this issue.

The mutation in *SEC63*, *sec63-1*, which abolished glycopeptide export and reduced misfolded protein export from the ER, interferes with recruitment of the ER-luminal chaperone BiP to the Sec61 channel (41). BiP promotes posttranslational protein import into the yeast ER by cycles of binding and release of the translocating nascent chain, acting as a molecular ratchet (42); therefore, both mutants in the gene encoding BiP, *KAR2*, and in *SEC63*, are severely defective in protein import into the ER (43). BiP function is also required for misfolded protein export from the ER, but its role in this process is less clear (15, 44). BiP has no measurable affinity for peptides smaller than four amino acids, does not bind to glycosylation acceptor tripeptides, and preliminary experiments with *kar2* mutant microsomes suggest that it is not involved in glycopeptide export from the ER (refs. 34, 35, and 45; K.R., unpublished work). Thus, the Sec63p requirement in glycopeptide export is most likely independent of its BiP recruitment function. A possible explanation for our observations is that in *sec63-1* membranes, a significant fraction of the Sec61 channels may be sequestered in dysfunctional Sec complexes with mutant Sec63p, and thus the number of channels

available for export across the ER membrane may be reduced. This may also be the reason for the decreased misfolded protein export from *sec63-1* membranes, which is independent of the other subunits of the Sec63 complex (14, 15). In this scenario, ER-luminal ATP may be required for the activity of an as-yet-unidentified ER protein involved in targeting glycopeptides to the translocon or gating of the translocon for peptide export.

Yeast microsomes represent a mixture of cellular membranes (41). To be able to monitor ER-specific processes, it is therefore necessary to design a substrate that can receive ER-specific modifications, such as signal peptide cleavage or N-glycosylation. An N-glycosylation acceptor site engineered into synthetic TAP substrates allowed monitoring of TAP-mediated peptide transport into the ER lumen by lectin precipitation of the translocated peptides (5, 6). Introduction of glycosylation acceptor tripeptides into a yeast cell-free system designed to monitor ER translocation, and ER-to-Golgi transport of secretory proteins led to the first observation of active retrograde transport across the ER membrane (11). Here we have used a glycosylation acceptor peptide with membranes from mutant yeast strains to demonstrate the involvement of the Sec61 channel in glycopeptide transport across the ER membrane to the cytosol. Given that the translocon can export both glycosylated and unglycosylated polypeptides, we consider it likely that the Sec61 channel is responsible for export of oligopeptides from the ER in general, whether glycosylated or not, and thus plays a pivotal role in both protein and peptide homeostasis in the secretory pathway.

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