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Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation

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Degradation of misfolded secretory proteins has long been assumed to occur in the lumen of the endoplasmic reticulum (ER). Recent evidence, however, suggests that such proteins are instead degraded by proteasomes in the cytosol, although it remains unclear how the proteins are transported out of the ER. Here we provide the first genetic evidence that Sec61p, the pore-forming subunit of the protein translocation channel in the ER membrane, is directly involved in the export of misfolded secretory proteins. We describe two novel mutants in yeast Sec61p that are cold-sensitive for import into the ER in both intact yeast cells and a cell-free system. Microsomes derived from these mutants are defective in exporting misfolded secretory proteins. These proteins become trapped in the ER and are associated with Sec61p. We conclude that misfolded secretory proteins are exported for degradation from the ER to the cytosol via channels formed by Sec61p.

Keywords: ER degradation/protein translocation/secretion/translocon/yeast

Introduction

Secretory proteins are targeted to and translocated across the endoplasmic reticulum (ER) membrane through a channel formed by the evolutionarily conserved Sec61 protein complex (Andrews and Johnson, 1996; Hanein *et al.*, 1996). Subsequently, proteins undergo folding and may acquire covalent modifications in the ER lumen prior to packaging into transport vesicles (Hurtley and Helenius, 1989). In yeast, the Sec61 complex consists of an essential polytopic transmembrane protein, Sec61p, and two smaller subunits, Sbh1p and Sss1p (Deshaies and Schekman, 1987; Esnault *et al.*, 1993; Panzner *et al.*, 1995). During protein translocation, Sec61p is in constant contact with the translocating chain, suggesting that it is the major constituent of the protein translocation channel (Mothes *et al.*, 1994). The contributions of Sbhp and Sss1p to the structure of the translocation pore are less clear. Two additional genes encoding transmembrane ER proteins *SEC62* and *SEC63* were identified in genetic screens for protein translocation mutants in yeast (Deshaies and Schekman, 1987; Rothblatt *et al.*, 1989). The corresponding proteins, Sec62p and Sec63p, are found in a complex with two other proteins, Sec71p and Sec72p (Sec63p complex; Deshaies *et al.*, 1991). At the ER membrane in yeast, a pre-secretory protein initially interacts with proteins of the the Sec63p complex and is transferred to the Sec61 channel in an ATP-dependent reaction (Müsch *et al.*, 1992; Sanders *et al.*, 1992; Lyman and Schekman, 1997). The translocation channel itself is gated and will open only in the presence of a functional signal sequence (Jungnickel and Rapoport, 1995).

Upon entry into the ER lumen, the signal sequence of a pre-secretory protein is cleaved, oligosaccharyl transferase glycosylates the appropriate sites and chaperones aid protein folding and oligomerization (Hurtley and Helenius, 1989). Failure to perform any of these steps results in retention of the secretory protein in the ER and one of two possible fates: protein aggregation or degradation (Klausner and Sitia, 1990). Recent evidence suggests that the degradation takes place in the cytosol rather than in the ER and is dependent on large cytosolic proteolytic complexes, the proteasomes, which are responsible for most of the protein turnover in the cytosol (Hiller *et al.*, 1996; Hilt and Wolf, 1996; Werner *et al.*, 1996; Wiertz *et al.*, 1996). A recently developed cell-free assay reproduces export from the ER and degradation of a misfolded secretory protein *in vitro* (McCracken and Brodsky, 1996). In this assay, cytosol derived from mutants in the chymotryptic proteasome subunits *pre1* and *pre2* promotes export, but degradation is substantially diminished (Werner *et al.*, 1996).

The export route of misfolded soluble proteins from the ER is unknown, but recently Wiertz and colleagues demonstrated that a transmembrane protein destined for degradation in the ER of virus-infected mammalian cells is transiently associated with Sec61β, the mammalian equivalent of Sbh1p, suggesting an involvement of the Sec61 channel in export (Wiertz *et al.*, 1996). We have isolated two new mutants in *sec61* that are cold-sensitive for protein import into the ER in both intact yeast cells and in a cell-free system. At the permissive temperature for protein import, microsomes derived from the mutants are defective in exporting a misfolded secretory protein that now remains trapped in the ER and is associated with Sec61p. We conclude that misfolded secretory proteins are exported for degradation from the ER to the cytosol via channels formed by Sec61p.

Results

Isolation of new conditional sec61 alleles

SEC61 was identified originally in yeast as a temperature-sensitive mutation defective in protein translocation into the ER (Deshaies and Schekman, 1987; Stirling *et al.*,

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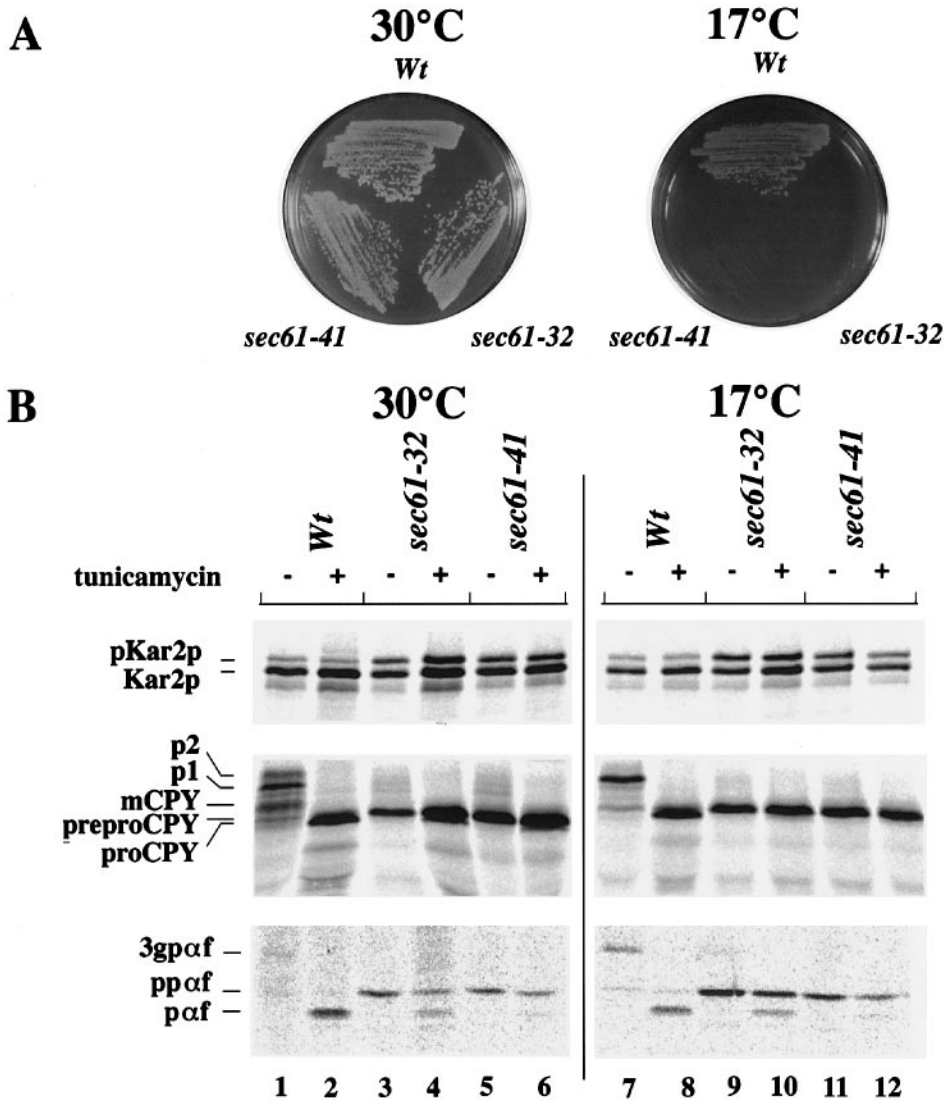

Fig. 1. *sec61-32* and *sec61-41* are deficient for secretory protein translocation into the ER. (**A**) *sec61-32* and *sec61-41* are cold-sensitive for growth. Wild-type and mutant cells were grown on YPD for 3 days at 30°C or 5 days at 17°C. (**B**) *sec61-32* and *sec61-41* are deficient for protein translocation into the ER *in vivo*. Wild-type and mutant cells were pulse-labelled at 30 or 17°C in the presence or absence of 10 μg/ml tunicamycin as described in Materials and methods, and secretory proteins were immunoprecipitated. The positions of the signal sequence-containing precursor forms (pKar2p, preproCPY, ppαf), signal-cleaved, unglycosylated proteins (Kar2p, proCPY, pαf) and of signal-cleaved glycosylated forms (p1, p2, mCPY, 3gpαf) are indicated. The band just below mature Kar2p is unrelated (Feldheim *et al.*, 1993).

1992). Two previously isolated alleles, *sec61-2 and sec61-3*, affect the stability of the Sec61 protein in yeast (Sommer and Jentsch, 1993), but do not have severe translocation defects when analysed *in vitro*, which limits their use for functional analysis of Sec61p in the translocation process. In order to gain a better understanding of the role of Sec61p in ER translocation, we isolated new mutants in *SEC61*. *SEC61* was mutagenized *in vitro* by hydroxylamine treatment, cloned into a *CEN/LEU2* plasmid, and introduced into a haploid yeast strain containing the wild-type *SEC61* on a *URA3* plasmid. Following plasmid shuffling (Sikorski and Boeke, 1991), we isolated two new mutant alleles, *sec61-32* and *sec61-41*, that rendered the cells cold-sensitive for growth (Figure 1A), but did not affect the ability to form colonies in the temperature range from 23 to 38°C. Both mutant strains, however, had a longer generation time of 140 min in rich medium (YPD) at 30°C compared with 90 min for the wild-type. The mutations did not affect the level of expression of Sec61p or other members of the translocation complex (data not shown). We sequenced the new *sec61* alleles and found that in both cases the mutations were single G to A changes, as expected for hydroxylamine mutagenesis (*sec61-32* at base 894, *sec61-41* at base 845). These base changes resulted in a cysteine to tyrosine substitution at amino acid position 150 for *sec61-32* and a valine to isoleucine change at position 134 for *sec61-41*.

To assess the translocation defects in the mutant cells, we examined the biogenesis of the ER resident protein Kar2p, the vacuolar protease carboxypeptidase Y (CPY) and the mating pheromone precursor prepro-alpha-factor (ppαf). Wild-type and mutant cells were pulse-labelled with [^{35}S]methionine/cysteine in the absence or presence of the glycosylation inhibitor tunicamycin followed by immunoprecipitation. Kar2p is translated with a signal sequence that is cleaved upon translocation into the ER.

The Kar2p precursor (pKar2p) was detected in both mutant and wild-type cells radiolabelled at 30°C (Figure 1B, lanes 1–6); however, the mutants accumulated significantly more pKar2p (~30% versus 4% in wild-type). pKar2p accumulation was increased to 45% at 17°C in *sec61* mutant cells, but no change in precursor accumulation was observed in wild-type cells (Figure 1B, lanes 7–12).

CPY is synthesized as a prepro-protein whose signal sequence is processed upon entering the ER lumen (Stevens *et al.*, 1982). ProCPY is *N*-glycosylated in the ER to the p1 form. Addition of outer chain mannose residues in the Golgi converts p1 to p2 CPY. In the vacuole, p2 CPY is processed by a vacuolar protease to the smaller mature CPY (mCPY), which in our gel system migrates just above preproCPY (Figure 1B, lane 1). In the absence of tunicamycin, wild-type cells contained p1, p2 and mCPY (Figure 1B, lane1). Tunicamycin inhibits glycosylation as well as transport and, therefore, only the signal-cleaved proCPY could be detected in wild-type cells treated with 10 μg/ml tunicamycin (Figure 1B, lane 2). At 30°C, *sec61-32* and *sec61-41* mutant cells accumulated predominantly the cytosolic preproCPY (Figure 1B, lanes 3 and 5), but a small fraction of preproCPY was still translocated and processed, as seen by the appearance of the higher mobility proCPY band in the cells treated with tunicamycin (Figure 1B, lanes 4 and 6). At 17°C, vesicular transport from the endoplasmic reticulum is slowed down, thus wild-type cells contained mostly p1 CPY (Figure 1B, lane 7). We observed a complete translocation block in both *sec61-32* and *sec61-41* at the restrictive temperature, resulting in the accumulation of preproCPY (Figure 1B, lanes 9–12).

Upon translocation into the ER, the signal sequence of ppαf is cleaved, yielding proalpha-factor (pαf) which becomes core-glycosylated at three asparagine residues (3gpαf). From wild-type cells, 3gpαf can be immunoprecipitated only at 17°C, and not at 30°C (Figure 1B, lanes 1 and 7), because the half-time for transport to the Golgi where it is cleaved to smaller peptides is shorter than the pulse time. Both mutants accumulated ppαf at both temperatures, but accumulation was more pronounced at 17°C. In *sec61-32* cells labelled in the presence of tunicamycin, a fraction of ppαf was still translocated and processed to pαf (Figure 1B, lanes 4 and 10), whereas the translocation block for ppαf in *sec61-41* cells was almost complete (Figure 1B, lanes 6 and 12). Taken together, our data show that *sec61-32* and *sec61-41* cells are deficient in the translocation of a variety of precursor proteins *in vivo*.

sec61-32 and sec61-41 membranes are cold-sensitive for protein translocation in vitro

In order to test the effect of *sec61-32* and *sec61-41* on post-translational protein translocation *in vitro*, we prepared microsomes from wild-type (*SEC61*) and mutant cells grown at the permissive temperature (30°C). We used non-saturating, equal amounts of microsomes and radiolabelled, *in vitro* translated ppαf as a translocation substrate. Translocation was measured by lectin precipitation of translocated, core-glycosylated 3gpαf and scintillation counting. In our cell-free system, the optimum temperature for protein translocation into wild-type microsomes was 24°C (*SEC61*, Figure 2). Microsomes prepared

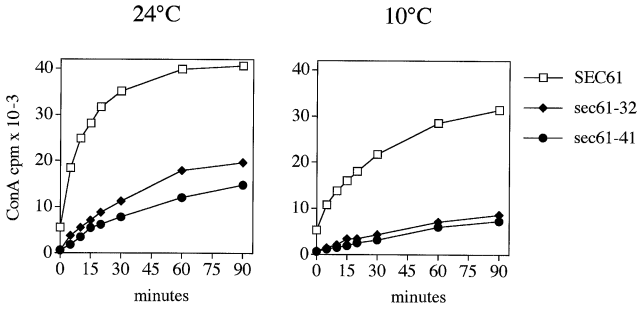

Fig. 2. *sec61-32* and *sec61-41* microsomes are deficient for protein translocation *in vitro*. Radiolabelled *in vitro* translated wild-type ppαf was translocated into microsomes derived from *SEC61* (RSY1293), *sec61-32* (RSY1294) or *sec61-41* (RSY1295) cells at 24 or 10°C in the presence of ATP and a regenerating system. Translocated protein was quantified by concanavalin A precipitation and scintillation counting. All samples were assayed in duplicate.

from the mutant cells were partially deficient for protein translocation at 24°C (Figure 2, 24°C): *sec61-41* microsomes translocated 40% of the amount of ppαf imported into wild-type membranes, whereas *sec61-32* membranes translocated up to 54% of wild-type (Figure 2, 24°C). These results indicate that the more severe translocation defect seen in *sec61-41* cells *in vivo* could be reproduced for ppαf in our cell-free system (compare Figure 1B, lanes 4 and 6). We used 10°C as the restrictive temperature for protein translocation *in vitro* (Figure 2, 10°C). Both mutants were severely defective at the low temperature, translocating respectively 28% (*sec61-32*) or 22% (*sec61-41*) of the amount of ppαf imported into wild-type membranes in 90 min (Figure 2, 10°C). We conclude that *sec61-32* and *sec61-41* are cold-sensitive for protein import into the ER both in intact cells and in isolated microsomes.

Sec61p is involved in export of misfolded secretory proteins from the ER to the cytosol

An increasing body of evidence suggests that 'ER degradation' is a misnomer and that misfolded secretory proteins and transmembrane proteins are exported from the ER to the cytosol and degraded by the multicatalytic cytosolic proteasomes (Hiller *et al.*, 1996; Werner *et al.*, 1996; Wiertz *et al.*, 1996). Recently, McCracken and Brodsky developed a cell-free assay for export and degradation of a mutated form of the secretory protein ppαf that has its three glycosylation site asparagines changed to glutamines (pΔgpαf) and is degraded after entry into the ER *in vivo* and *in vitro* (Caplan *et al.*, 1991; McCracken and Brodsky, 1996). We used this cell-free system to ask whether membranes derived from our *sec61* mutants were deficient in export of misfolded Δgpαf to the cytosol for degradation.

In vitro translated, signal sequence-containing pΔgpαf was translocated into microsomes post-translationally, resulting in signal-cleaved Δgpαf present in the washed membranes (Figure 3A, $t = 0$) that is not accessible to added protease (data not shown). Also present in these samples is the non-specifically membrane-associated precursor (pΔgpαf) which is resistant to washes, but is partially accessible to exogenously added protease (see Figure 3D). In wild-type microsomes, Δgpαf, but not the glycosylated wild-type protein (not shown), was degraded at 24°C in the presence of ATP and wild-type (PRE)

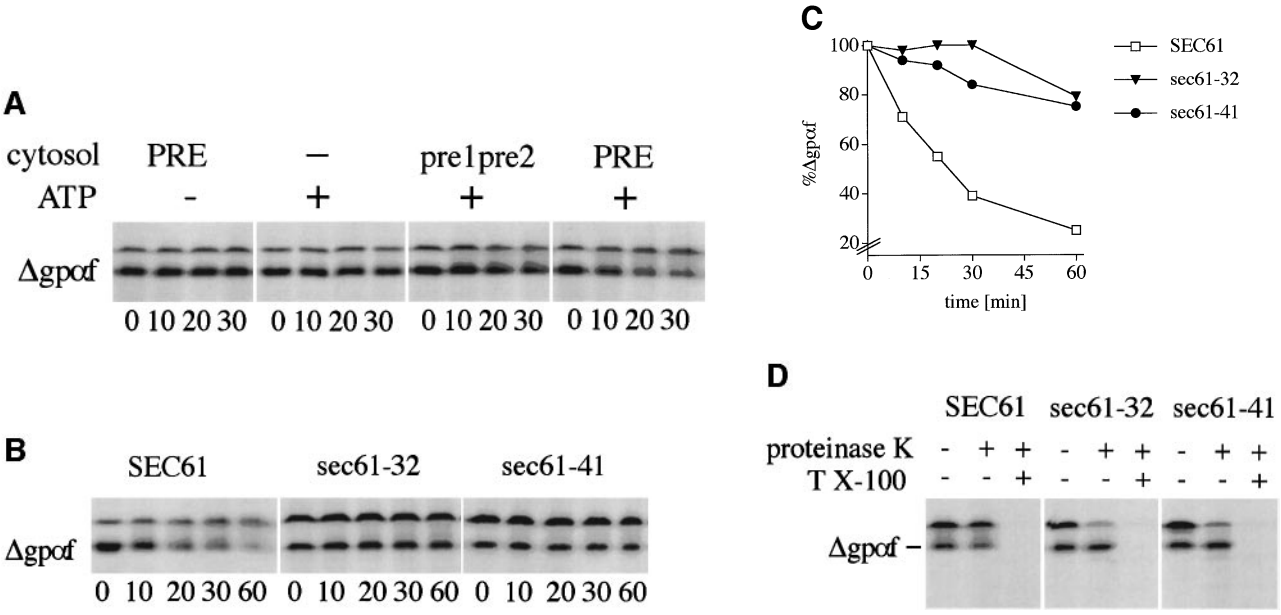

Fig. 3. *sec61-32* and *sec61-41* membranes are deficient for 'ER degradation' *in vitro*. (**A**) 'ER degradation' *in vitro* is dependent on ATP and cytosolic proteasomes. Wild-type (RSY255) microsomes (2 μl OD_{280} = 30 per lane) were loaded with Δgpαf and subsequently incubated in the presence or absence of 6 mg/ml wild-type cytosol (PRE, from WCG4a) or proteasome-deficient cytosol (pre1pre2, from WCG4-11/22a) and in the presence or absence of ATP and an ATP-regenerating system, as indicated at 24°C. Incubations were terminated at the indicated time points (in minutes) by precipitation with trichloroacetic acid and samples analysed on 18% polyacrylamide/4 M urea gels followed by autoradiography. The lower mobility band is pΔgpαf associated with the cytosolic face of the microsomal membranes. (**B**) *sec61-32* and *sec61-41* membranes are deficient for 'ER degradation' *in vitro*. Wild-type (RSY1293) or mutant membranes (RSY1294 and RSY1295) were loaded with Δgpαf and incubated in the presence of ATP and 6 mg/ml PRE cytosol at 24°C for the indicated period of time (in minutes). Reactions were terminated and samples analysed as above. Note that an increased amount of pΔgpαf is associated with microsomes derived from the mutants. (**C**) The Δgpαf bands from three different degradation experiments (performed as in B) were quantified using a phosphorimager (BioRad). Variation at each time point was <10%. (**D**) Δgpαf in *sec61-32* and *sec61-41* microsomes is protease-protected. Degradation reactions containing Δgpαf-loaded *SEC61* (RSY1293), *sec61-32* (RSY1294) or *sec61-41* (RSY1295) membranes were incubated with ATP and 6 mg/ml PRE cytosol for 20 min at 24°C and then transferred to ice. Proteinase K (0.1 mg/ml final concentration) or proteinase K and Triton X-100 (1% final concentration) were added and samples incubated on ice for 30 min. Reactions were terminated by addition of PMSF and samples processed as described above. A fraction of pΔgpαf is aggregated and therefore resistant to proteinase K in the absence of detergent.

cytosol with a $t_{1/2}$ of 15 min (Figure 3A). If cytosol from a strain with mutations in two catalytic subunits of the proteasome (*pre1pre2*) was used in the assay, Δgpαf was degraded more slowly ($t_{1/2}$ = 45 min; Figure 3A). These findings are in full agreement with previously published data (McCracken and Brodsky 1996; Werner *et al.*, 1996) and indicate that mutated secretory proteins such as Δgpαf are indeed exported from the ER lumen to the cytosol prior to degradation.

We next investigated whether the protein translocation channel formed by the Sec61 complex is involved in this export process. pΔgpαf was translated *in vitro* and imported into equal amounts of *sec61-32* or *sec61-41* mutant or isogenic wild-type microsomes (SEC61) at 24°C for 50 min. The washed membranes were then incubated with ATP and 6 mg/ml wild-type cytosol at 24°C for increasing periods of time. Δgpαf was exported from *SEC61* wild-type microsomes and degraded with a $t_{1/2}$ of ~20 min (Figure 3B and C). In contrast, Δgpαf was exported from *sec61-41* microsomes with a $t_{1/2}$ of >60 min (Figure 3B and C), and there was virtually no export of Δgpαf from *sec61-32* microsomes in the first 30 min (Figure 3B and C). In order to analyse whether the Δgpαf in reactions containing *sec61-32* or *sec61-41* microsomes was retained in the microsomal lumen, after 20 min of export at 24°C we transferred the reactions to ice, and digested the samples with proteinase K in the presence or absence of detergent. In reactions containing wild-type membranes, ~50% of Δgpαf are protease-sensitive after 20 min of incubation, suggesting that the limiting step in degradation is the proteasome activity (Figure 3D). In contrast, Δgpαf that remains in reactions containing *sec61-32* or *sec61-41* mutant membranes is fully protected from digestion with proteinase K, indicative of its retention in the ER lumen. Our data demonstrate that *sec61-32* and *sec61-41* membranes, which are cold-sensitive for protein import, are deficient for misfolded protein export at the permissive temperature for import. These findings strongly suggest that the export of Δgpαf is mediated by Sec61p.

sec61, but not sec62 or sec63 mutants are defective in misfolded protein export

A temperature-sensitive mutant allele of *SEC61*, *sec61-3* (Stirling *et al.*, 1992), and temperature-sensitive mutations in two other genes encoding proteins required for post-translational protein import into the yeast ER, *sec62-1* and *sec63-1* (Rothblatt *et al.*, 1989), were isolated previously. In order to test whether the export defect observed in *sec61-32* and *sec61-41* was specific for *SEC61*, we prepared microsomes from *sec61-3*, *sec62-1* and *sec63-1* cells grown at the permissive temperature (24°C) for use in the *in vitro* degradation assay described above. Equal amounts of membranes were used in all reactions, but to compensate for the strong translocation defects in the mutants all panels showing reactions with mutant microsomes were exposed twice as long as the wild-type

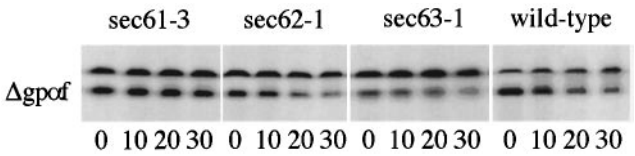

Fig. 4. *sec61-3*, but not *sec62-1* or *sec63-1* membranes, are deficient for 'ER degradation' *in vitro*. Wild-type (RSY255), *sec61-3* (CSY150), *sec62-1* (RSY529) and *sec63-1* (RSY155) membranes were loaded with Δgpαf and incubated in the presence of ATP and 6 mg/ml PRE cytosol at 24°C for the indicated period of time (in minutes). Reactions were terminated and samples analysed as above. Equal amounts of membranes were used, but the part of the figure showing the mutant membranes was exposed for twice as long as the wild-type. Note that an increased amount of pΔgpαf is associated with mutant microsomes.

(Figure 4). As shown in Figure 4, *sec62-1* and *sec63-1* microsomes exported Δgpαf for degradation, although with slightly slower kinetics than wild-type membranes ($t_{1/2}$ = 15 min wild-type; 20 min *sec62-1*; 30 min *sec63-1*). In contrast, in reactions containing *sec61-3* microsomes, export and degradation of Δgpαf was strongly retarded and 92% of Δgpαf were still present after 30 min of incubation (Figure 4). Like *sec61-32* and *sec61-41*, *sec61-3* membranes exhibit a pronounced export defect at the temperature that is permissive for import (24°C). These data indicate that the export defect is specific for *sec61* mutants.

Misfolded secretory proteins destined for export interact with Sec61p

During secretory protein import into the ER, translocation intermediates can be chemically cross-linked to Sec61p (Müsch *et al.*, 1992; Sanders *et al.*, 1992; Lyman and Schekman, 1997). We used the amino group-reactive, thiol-cleavable cross-linker dithiobis-(sulfosuccinimidyl-propionate) (DSP) to examine whether substrates for export from the ER to the cytosol interacted with Sec61p directly and whether the mutations in *SEC61* had any effect on these interactions. At the end of 50 min translocation reactions at 24°C in the presence of ATP, an ATP-regenerating system and pΔgpαf or ppαf, we treated *SEC61* wild-type or mutant microsomes with DSP. Subsequently, Sec61p and associated proteins were immunoprecipitated from the detergent-solubilized membranes with affinity-purified anti-Sec61p antibodies. The cross-links were cleaved with the reducing agent dithiothreitol (DTT), and Sec61p-associated proteins analysed by electrophoresis on 18% acrylamide/4 M urea SDS gels.

Under ER import conditions *in vitro*, export and degradation of misfolded secretory proteins are limited by the low concentration of cytosol. Thus, misfolded proteins in the ER lumen might accumulate at a step just prior to export. Indeed, we found that after signal sequence cleavage, Δgpαf, but not wild-type glycosylated 3gpαf, could be cross-linked to Sec61p (Figure 5A, lanes 1–3 and 7–9). The co-precipitation of Δgpαf with Sec61p was entirely dependent on the presence of the cross-linker (Figure 5A, compare lanes 1–3 and 4–6) and was increased in *sec61* mutant membranes compared with wild-type (Figure 5A, lanes 1–3, 1.9× for *sec61-32*, 1.5× for *sec61-41*) where it accounted for ~1–2% of total Δgpαf present in the sample. We conclude that misfolded secretory proteins associate with the Sec61 channel prior to export, whereas

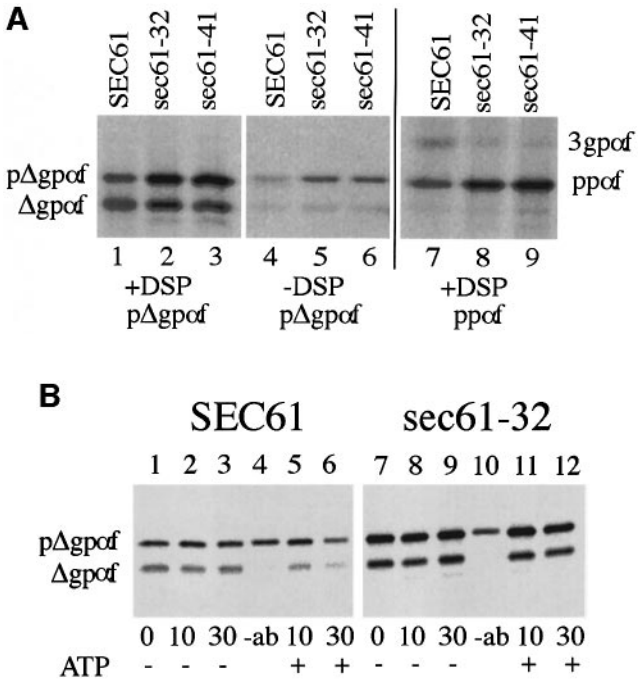

Fig. 5. Interactions of secretory precursors and translocated signal-cleaved secretory proteins with Sec61p in wild-type and *sec61* mutant membranes. (**A**) pΔgpαf (lanes 1–6) or ppαf (lanes 7–9) was translocated into wild-type or mutant microsomes as indicated. Directly after termination of the translocation reaction, DSP cross-linking was performed as described in Materials and methods (lanes 1–3 and 7–9: 2.5 mM DSP; lanes 4–6: no DSP control) followed by solubilization of the membranes and Sec61p immunoprecipitation. Cross-links were cleaved by addition of DTT prior to electrophoresis. The positions of precursor proteins (pΔgpαf, ppαf) and translocated signal-cleaved (Δgpαf) and glycosylated forms (3gpαf) are indicated. The increased amounts of pΔgpαf and ppαf associated with mutant Sec61p are probably a result of the increased amounts of precursor non-specifically associated with the mutant membranes (see previous figures) rather than translocation intermediates. (**B**) Following pΔgpαf translocation, *SEC61* or *sec61-32* membranes were incubated in the presence of 6 mg/ml cytosol and the presence or absence of ATP and the regenerating system for 0, 10 or 30 min at 24°C prior to the addition of 2.5 mM DSP. Cross-linking was done as described in Materials and methods, followed by solubilization of the membranes and Sec61p immunoprecipitation. Cross-links were cleaved by addition of DTT prior to electrophoresis. –ab: no antibody control for immunoprecipitation. A fraction of pΔgpαf that was co-precipitated with Sec61p was non-specifically associated with the protein A–Sepharose beads, as the binding did not require the presence of the antibodies (compare lanes 1 and 4, 7 and 10). Note that aggregated pΔgpαf on the cytosolic face of the microsomes is partially degraded upon addition of cytosol and ATP (lanes 6 and 12) and thus a lower amount is found associated with Sec61p.

correctly folded secretory proteins do not interact with Sec61p once translocation is completed.

We subsequently analysed whether Δgpαf associated with Sec61p is an export intermediate. Microsomes isolated from wild-type cells or *sec61-32* cells (which exhibit the strongest defect in degradation) were loaded with Δgpαf as described above and subsequently incubated in the absence or presence of ATP and cytosol at 24°C. After 0, 10 or 30 min at 24°C, the microsomes were treated first with DSP and then solubilized with detergent, the Sec61p-associated material immunoprecipitated, and the cross-links cleaved with reducing agent. In the presence of cytosol, but in the absence of ATP—conditions that do not promote export and degradation (Figure 3A)—Δgpαf

remained associated with Sec61p in both mutant and wild-type membranes (Figure 5B, lanes 1–3 and 7–9). Similarly, the presence of ATP alone was not sufficient to cause dissociation of Δgpαf from Sec61p (data not shown). In the presence of cytosol and ATP—conditions that allow export and degradation—Δgpαf dissociated from wild-type Sec61p (Figure 5B, lanes 5 and 6) and presumably was released into the cytosol for degradation. In contrast, in *sec61-32* microsomes, Δgpαf remained associated with Sec61p (Figure 5B, lanes 11 and 12). Our gel system resolves polypeptide chains with a size difference of ⩾10 amino acids; however, as shown in Figure 3D, the export substrate was fully protease-protected in *sec61* mutant membranes and no partially proteolysed intermediates could be detected. Taken together, our data indicate that Δgpαf found associated with Sec61p in the experiments shown in Figure 5 is fully translocated. Thus, the mutant Sec61p in *sec61-32* and *sec61-41* membranes is unable to mediate export of Δgpαf, and the Δgpαf in these microsomes is associated with the Sec61p on the lumenal face of the ER membrane.

Discussion

We have provided both genetic and biochemical evidence for a direct role for Sec61p in the export of a misfolded secretory protein from the lumen of the ER to the cytosol. This is an important novel function of Sec61p in addition to its well established role in import. Membranes derived from two new cold-sensitive mutants in *sec61* (*sec61-32* and *sec61-41*) and a previously characterized temperature-sensitive mutant (*sec61-3*) were deficient in an *in vitro* assay that reproduces export from the ER for proteasome-dependent degradation in the cytosol. Mutations in two other genes encoding proteins required for secretory protein import into the ER (*sec62-1* and *sec63-1*) did not strongly affect export. After translocation into *sec61* mutant microsomes, the export substrate was protected from protease digestion, indicating that the import reaction was completed and, in contrast to a correctly glycosylated secretory protein, it specifically associated with Sec61p. The misfolded secretory protein was released from wild-type Sec61p, exported and degraded in the presence of ATP and cytosol, but remained Sec61p-associated and protease-protected in *sec61* mutant membranes.

The mutations in *sec61-32* and *sec61-41* map to the ER lumenal ends of transmembrane domains IV and III of Sec61p, respectively (Wilkinson *et al.*, 1996), suggesting an important role for this region in both protein import and export. Cells harbouring these mutations are defective in protein export at the permissive temperature for growth and protein import.

This suggests that misfolded protein export from the ER to the cytosol is not an essential function, consistent with reports on other yeast mutants which are deficient in 'ER degradation', but viable (Hampton *et al.*, 1996; Hiller *et al.*, 1996; Knop *et al.*, 1996). At lower temperatures, *sec61-41* and *sec61-32* mutations may affect Sec61p structure or mobility in the membrane.

The import defect in *sec61-41* membranes is more severe than the export defect and vice versa for *sec61-32* (Figures 1, 2, 3B and C). In addition, microsomes derived from *sec62-1* or *sec63-1* cells, which are more import deficient than any of the *sec61* mutant membranes, are only marginally affected in export (Figure 4). Taken together, these observations argue strongly in favour of a direct role for Sec61p as a part of the export machinery and argue against the possibility that the export defect is indirectly caused by a reduced protein translocation activity in the *sec61* mutants. In wild-type ER, export to the cytosol is temperature-dependent and severely slowed down at temperatures below 20°C (data not shown); therefore, it was not possible to investigate whether the export defect in *sec61-41* was stronger at lower temperatures. Cross-linking experiments showed that signal-cleaved misfolded Δgpαf, but not properly glycosylated and folded 3gpαf, is in association with wild-type and mutant Sec61p. These data demonstrate a direct role for Sec61p in misfolded protein export. Our data are consistent with recent work from Wiertz and colleagues who demonstrated that major histocompatibility complex (MHC) class I heavy chains destined for degradation reassociate with the translocation channel, and who isolated export intermediates that interacted with both Sec61β and the proteasome in cytomegalovirus-infected mammalian cells (Wiertz *et al.*, 1996).

Export of a signal-cleaved misfolded secretory protein is difficult to explain with our current knowledge of protein translocation. During import, the translocation channel itself recognizes and requires the presence of a functional signal sequence (Jungnickel and Rapoport, 1995). After signal cleavage, opening of the translocation channel from the lumenal side must be triggered by an as yet unknown mechanism, possibly by the interaction with both the misfolded secretory protein and the chaperone involved in the folding attempt. In this context, it is interesting that membranes from cells carrying the *sec63-1* mutation, which is defective in the recruitment of BiP/Kar2p to the translocation machinery during post-translational protein import into the yeast ER (Lyman and Schekman, 1995), are not strongly deficient for misfolded secretory protein export (Figure 4). The possible role of Kar2p in protein export from the ER remains to be considered. The use of the same translocation channel for both import and export seems an economical solution for bidirectional protein trafficking across the ER membrane. In addition, this arrangement might allow for designation of export substrates before their import into the ER is completed. Chaperone proteins of the quality control mechanism in the ER, such as calnexin and Kar2p, are positioned along with the glycosylation machinery very close to the translocon (Andrews and Johnson, 1996; McCracken and Brodsky, 1996). Although Δgpαf was fully translocated to the lumenal side of the translocon (not accessable to exogenously added proteinase K), our data do not indicate whether dissociation from Sec61p had occurred prior to export. Thus, either Δgpαf is released into the ER lumen, but does not pass quality control and therefore reassociates with Sec61p, or chaperones engage prior to release from the channel. In the first case, the gating of the translocon must be regulated from the lumenal side by a mechanism independent of the signal sequence. In the second scenario, entry of undesired polypeptides into the secretory pathway would be prevented at a very early stage, requiring no additional gating mechanism. In the case of the transmembrane MHC class I

Table I. Yeast and bacterial strains

Strain	Genotype	Source or reference
Saccharomyces cerevisiae		
RSY633	*MATα can 1-100 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 sec61::HIS3* [pDF40 (*Hin*dIII–*Sty*I fragment of *SEC61* in pRS316)]	this study
RSY1293	*MATα can 1-100 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 sec61::HIS3* [pDQ1]	this study
RSY1294	as *RSY1293* except [*psec61-32*]	this study
RSY1295	as RSY1293 except [p*sec61-41*]	this study
RSY255	*MATα leu2-3,-112 ura3-52*	Stirling *et al.* (1992)
CSY150	*MATα leu2-3,-112 trp1-1 ura3-52 sec61-3*	Stirling *et al.* (1992)
RSY529	*MATα leu2-3,-112 his4-619 ura3-52 sec62-1*	Rothblatt *et al.* (1989)
RSY155	*MATα leu2-3, -112 ura3-52 ade2-1 pep4-3 sec63-1*	Rothblatt *et al.* (1989)
WCG4a	*MAT***a** *leu2-3,-112 ura3 his3-11,-15*	Hiller *et al.* (1996)
WCG4-11/22a	*pre1-1 pre2-2*, same as WCG4a	Hiller *et al.* (1996)
Escherichia coli		
Xl1-blue	*recA1 lac*$^-$ *endA1 gyrA96 thi hsdR17 supE44 relA* [F′ *proAB lac17 lacZΔm5* Tn*10*]	(Stratagene)

heavy chain, export to the cytosol can be envisaged as a simple reversal of import, mediated by the re-entry of the transmembrane domain into the translocation channel (Wiertz *et al.*, 1996).

We propose the following series of events for protein import and export through the Sec61 channel. Secretory proteins are targeted to the ER membrane via their N-terminal signal sequence. Recognition of the signal sequence by the translocation channel causes channel opening and initiation of translocation (Jungnickel and Rapoport, 1995). During and after translocation, secretory proteins fold with the aid of chaperones. After successful folding, these proteins are released from the chaperones and packaged into ER-to-Golgi transport vesicles. Failure to fold, for example in mutant secretory proteins, results in reassociation or prolonged association with the protein translocation channel, and export to the cytosol for degradation by the proteasome. In this model, targeting to and export from the ER are ATP- and cytosol-dependent; all intermediate steps require the presence of ATP only. We show that the new *sec61* mutants analysed here are defective at the permissive temperature in misfolded protein export, and defective in channel opening or initiation of translocation at the restrictive temperature. The *sec61* mutants that are specifically defective for export at a permissive temperature for import will be used in future experiments to decipher how the ER regulates incoming and outgoing traffic in what used to be regarded as a one-way road into the secretory pathway.

Materials and methods

Strains and growth conditions

The strains used in this study are listed in Table I. Media were purchased from Difco Inc. (Detroit, MI). Yeast cells were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) or synthethic media (SD) with the appropriate additions (Sherman, 1991). Media for plates were supplemented with 2% agar.

Strain construction

A six-histidine tagged version of *SEC61* was made by subcloning the 2.4 kb *Hin*dIII fragment containing *SEC61* into pUC119 (pDF42) and introducing an *NsiI* site at the start codon by recombinant PCR. An oligonucleotide linker encoding a six-histidine sequence was ligated into the *Nsi*I and *Xba*I sites. To allow the assessment of mutagenesis in *Escherichia coli*, we cloned the 1.3 kb kanamycin resistance cassette (Pharmacia, Uppsala, Sweden) into the *Eco*RI site, resulting in pMP6. To construct a low-copy-number yeast expression plasmid for plasmid shuffling, the *SEC61* coding sequence was subcloned as a *Hin*dIII–*Eco*RI fragment into the yeast shuttle vector YCpLac111 (Gietz and Sugino 1988) resulting in pDQ1. This plasmid fully complemented the temperature-sensitive phenotype of *sec61-3* and the chromosomal deletion of *sec61* in RSY633 upon plasmid shuffling on 5-fluoro-orotic acid (5-FOA) plates (Boeke *et al.*, 1987). pMP6 was mutagenized *in vitro* by hydroxylamine treatment as described by Rose and Fink (1987), except that incubation was for 1 h at 75°C. Upon transformation into *E.coli* and selection for ampicillin resistance, we found that 4% of the colonies were unable to grow on LB plus kanamycin. From 50 000 ampicillin-resistant colonies, the plasmid DNA was isolated and the *Xba*I–*Eco*RI fragment containing the coding sequence was subcloned into pDQ1 containing the *SEC61* promotor region. Upon transformation into *E.coli*, 40 000 colonies were obtained. The plasmids were isolated from four pools of 10 000 colonies each, and equal amounts were mixed and transformed into RSY633. Colonies (6000) were obtained on SD-Leu-His and subjected to plasmid shuffling on 5-FOA at 28°C. Colonies with a recessive cold-sensitive phenotype (no growth at 17°C after plasmid shuffling) were detected by replica-plating onto YPD. Plasmids from cold-sensitive colonies were isolated and rescreened, and designated p*sec61-32* and p*sec61-41*. The *sec61* sequence from these plasmids was subcloned, retested and sequenced.

Radiolabelling of cells and immunoprecipitation

Wild-type and mutant cells were grown to an OD_{600} of 2–3 at 30°C in complete minimal medium without methionine and cysteine, then diluted in the same medium to OD_{600} = 0.25 and further incubated at 30°C. When the cultures reached an OD_{600} = 1, 2 ml were transfered to either 30 or 17°C; tunicamycin was present at 10 μg/ml where indicated. After a 15 min pre-incubation, radiolabelling was initiated by the addition of [^{35}S]Promix (1200 Ci/mol Amersham, Arlington Heights IL, methionine and cysteine) to 20 μCi/OD_{600} of cells and incubation was continued for 15 min at the growth temperature. The cultures were then added to an equal volume of ice-cold 20 mM NaN_3/20 mM KF; protein extracts were prepared according to Doering and Schekman (1996) and secretory precursors were immunoprecipitated as described (Feldheim *et al.*, 1993) and analysed on 7.5% polyacrylamide gels (pKar2p, pCPY) or 18% polyacrylamide gels containing 4 M urea (ppαf).

Preparation of microsomes

Cells were grown in YPD at 30°C for RSY1293, RSY1294 and RSY1295 or 24°C for RSY255, CSY150, RSY529 and RSY155, and microsomes were prepared as described previously (Lyman and Schekman, 1995), except that spheroplasts were frozen at –80°C and thawed prior to homogenization. Microsomes were stored at OD_{280} = 30–40 in 20 or 50 μl aliquots at –80°C.

Preparation of cytosol

Cytosol was prepared from exponentially growing WCG4a and WCG4-11/22a cells (both strains from D.Wolf, University of Stuttgart) in YPD

at 30°C by liquid nitrogen lysis (Dunn and Wobbe, 1990). Cytosol was frozen in aliquots at 17–26 mg/ml protein and stored at –80°C.

Translocation assay

In vitro translated wild-type ppαf was translocated into wild-type or mutant microsomes at 10 or 24°C in the presence of ATP and an ATP-regenerating system as described previously (Lyman and Schekman, 1995). Equal amounts of microsomal protein were used for each time point. All samples were done in duplicate. Translocation was quantified by precipitation of glycosylated 3gpαf with concanavalin A (Pharmacia, Uppsala, Sweden) and scintillation counting (Lyman and Schekman, 1995).

ER degradation assay

ER degradation of the non-glycosylated form of pro-alpha-factor (Δgpαf) (Mayinger and Meyer, 1993) was assayed essentially as described previously (McCracken and Brodsky, 1996). Briefly, 20 µl of translocation reactions contained 2 µl of microsomes of OD_{280} = 30, B88 (20 mM HEPES, pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 250 mM sorbitol), ATP and a regenerating system (40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 1 mM ATP, 50 µM GDP-mannose) and 2 µl of *in vitro* translated, ^{35}S-labelled ppαf or pΔgpαf (500 000 c.p.m.). Translocation reactions were incubated for 50 min at 24°C, and the membranes were washed twice in B88. Membranes were resuspended in B88 containing ATP and the regenerating system and degradation reactions were started by adding cytosol to 6 mg/ml final concentration in a 20 µl/reaction final volume. Degradation reactions were incubated at 24°C for the indicated periods of time. The kinetics of export and degradation were similar at 24 and 30°C (data not shown), but mutant membranes were unstable during prolonged incubations at 30°C; therefore 24°C was chosen as the assay temperature. At the end of the incubation, samples were precipitated with trichloroacetic acid (TCA) and analysed after electrophoresis on 18% polyacrylamide–4 M urea SDS gels with a BioRad phosphorimager. For protease protection assays, at the end of the degradation incubation, samples were placed on ice for 5 min, then proteinase K (Boehringer Mannheim) was added to a final concentration of 0.1 mg/ml in the presence or absence of 1% Triton X-100. Protease digestion was performed for 30 min on ice, then stopped by addition of phenylmethylsulfonyl fluoride (PMSF; Sigma) to 40 mM final concentration and precipitation with TCA.

Cross-linking and immunoprecipitation

Washed microsomes (20 µl, 200 µg protein) loaded with Δgpαf as above were resuspended in 100 µl of B88, pH 7.4 (20 mM HEPES, pH 7.4, 150 mM potassium acetate, 5 mM magnesium acetate, 250 mM sorbitol) with or without 2× ATP mix (80 mM creatine phosphate, 0.4 mg/ml creatine phosphokinase, 2 mM ATP, 100 µM GDP-mannose). Cytosol (100 µl at 12 mg/ml) was added and DSP (Pierce) was added to 2.5 mM immediately or after 10 or 30 min at 24°C. Incubation was continued at 24°C for 20 min in the presence of DSP. Samples were transferred to ice, and ammonium acetate added to 400 mM. After 20 min on ice, samples were solubilized by adding SDS to 1% and heating to 65°C for 5 min. Immunoprecipitations were done with 10 µg of affinity-purified anti-Sec61p antibody (Lyman and Schekman, 1995).

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