

Molecular Chaperones in the Yeast Endoplasmic Reticulum Maintain the Solubility of Proteins for Retrotranslocation and Degradation

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Abstract. Endoplasmic reticulum (ER)-associated degradation (ERAD) is the process by which aberrant proteins in the ER lumen are exported back to the cytosol and degraded by the proteasome. Although ER molecular chaperones are required for ERAD, their specific role(s) in this process have been ill defined. To understand how one group of interacting luminal chaperones facilitates ERAD, the fates of pro- α -factor and a mutant form of carboxypeptidase Y were examined both in vivo and in vitro. We found that these ERAD substrates are stabilized and aggregate in the ER at elevated tem-

peratures when BiP, the luminal Hsp70 molecular chaperone, is mutated, or when the genes encoding the J domain-containing proteins Jem1p and Scj1p are deleted. In contrast, deletion of *JEM1* and *SCJ1* had little effect on the ERAD of a membrane protein. These results suggest that one role of the BiP, Jem1p, and Scj1p chaperones is to maintain luminal ERAD substrates in a retrotranslocation-competent state.

Key words: Jem1p • BiP • ERAD • molecular chaperone • protein translocation

Introduction

Newly synthesized secretory proteins translocate into the ER, and a quality control system ensures that only correctly folded and/or assembled proteins are transported from the ER to their ultimate destinations. However, misfolded or unassembled multimeric proteins are retained in the ER and may aggregate or be degraded (Ellgaard et al., 1999). A process that we termed ER-associated protein degradation (ERAD)¹ (McCracken and Brodsky, 1996; Werner et al., 1996) plays a central role in quality control by selectively degrading terminally misfolded or unassembled proteins (for review see Brodsky and McCracken, 1999; Plemper and Wolf, 1999). The ERAD of many soluble and membrane proteins requires the 26S proteasome located in the cytosol, suggesting that these proteins are retrotranslocated from the ER to the cytosol. In fact, much evidence indicates that the protein translocation channel, composed of the Sec61p complex in the ER membrane, mediates the retrotranslocation of ERAD substrates (for review see Römisch, 1999). This hypothesis was first proposed because an ERAD substrate in mammalian cells coprecipitates with Sec61p (Wiertz et

al., 1996). In accordance with this result, CPY*, a mutated form of vacuolar carboxypeptidase Y (CPY) that is degraded by ERAD (Hiller et al., 1996), was stabilized in *sec61* mutant yeast (Plemper et al., 1997), and the degradation of Δ Gp α F, an unglycosylated mutant form of the yeast α mating pheromone pro- α -factor (p α F), was inhibited in microsomes prepared from *sec61* strains (Pilon et al., 1997, 1998). Strains containing mutant alleles of *SEC61* that are proficient for protein import but are defective in the retrotranslocation process were isolated (Zhou and Schekman, 1999; Wilkinson et al., 2000), suggesting that these processes are mechanistically distinct.

BiP, an Hsp70 molecular chaperone in the yeast ER lumen, has also been shown to facilitate ERAD. BiP recognizes unfolded proteins and facilitates protein folding in the yeast ER (Simons et al., 1995). Stabilization of soluble ERAD substrates, such as CPY* and a variant of the α 1-protease inhibitor, A1PiZ, was observed in yeast cells containing temperature-sensitive mutations in *KAR2*, the gene encoding yeast BiP, and the degradation of Δ Gp α F was inhibited in microsomes prepared from temperature-sensitive *kar2* mutants (Plemper et al., 1997; Brodsky et al., 1999). BiP also drives posttranslational import into the ER by acting as a molecular ratchet (Matlack et al., 1999). Because some temperature-sensitive *kar2* mutants that are proficient for import show ERAD defects, the role of BiP in ERAD differs from its role in protein import (Brodsky

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¹Abbreviations used in this paper: CPY, carboxypeptidase Y; ERAD, ER-associated degradation; p α F, pro- α -factor; pp α F, prepro- α -factor.

et al., 1999). However, the molecular mechanisms underlying the function of BiP in ERAD are not clear.

Interestingly, the yeast ER contains a chaperone that is ~24% identical to BiP, known variably as Lhs1p (Craven et al., 1996), Cer1p (Hamilton and Flynn, 1996), or Ssi1p (Baxter et al., 1996). The translocation of some preproteins in cells lacking *LHS1* is impaired, and genetic interactions have been observed between this chaperone and BiP. Although a role for Lhs1p in stabilizing and refolding thermally denatured proteins in the ER has been demonstrated (Saris et al., 1997), it is unknown whether this chaperone plays a role in ERAD.

DnaJ-like proteins regulate the ATP-dependent reaction cycle of Hsp70 by interacting with Hsp70 via their conserved J domains (Kelley, 1998). The yeast ER contains three DnaJ-like proteins: Sec63p, Jem1p, and Scj1p (Sadler et al., 1989; Schlenstedt et al., 1995; Nishikawa and Endo, 1997). Sec63p is an essential membrane protein and interacts with BiP to drive the translocation of proteins into the ER lumen (Rothblatt et al., 1989; Brodsky and Schekman, 1993; Lyman and Schekman, 1995; Corsi and Schekman, 1997; Matlack et al., 1999). Jem1p and Scj1p are ER luminal proteins that are not essential for cell viability, but cells lacking both Jem1p and Scj1p are temperature sensitive, suggesting that their functions overlap (Nishikawa and Endo, 1997). The Δ *jem1* Δ *scj1* strain is proficient for protein import into the ER, but is defective for the transport of CPY*. Because the quality control system prevents the transport of defective proteins through the secretory pathway, Jem1p and Scj1p are likely involved in protein folding and assembly in the ER lumen (Silberstein et al., 1998). Scj1p interacts with BiP via its J domain (Schlenstedt et al., 1995) and in cooperation with BiP, Jem1p mediates nuclear membrane fusion during mating (Nishikawa and Endo, 1997; Brizzio et al., 1999). Thus, BiP may engage each of the three J domain-containing chaperones in the yeast ER to drive several cellular processes.

Because DnaJ-like proteins regulate Hsp70s, it is natural to suspect that Sec63p, Jem1p, and Scj1p are involved in ERAD. Although an increase in the half-life of CPY* in the ER was observed using a *sec63-1* mutant, this effect is marginal when compared with the defect observed in *sec61* mutants (Plempner et al., 1997). Similar results were observed for the in vitro degradation of Δ Gp α F using microsomes prepared from the *sec63-1* mutant (Pilon et al., 1997).

In this study, we report that Jem1p and Scj1p, the soluble DnaJ-like proteins in the ER lumen, are involved in the ERAD of CPY* and Δ Gp α F. CPY* and Δ Gp α F aggregate in a temperature-sensitive *kar2* mutant and the Δ *jem1* Δ *scj1* strain at elevated temperature, and once aggregation occurs, the ERAD of these proteins is significantly impaired. We propose that BiP cooperates with the soluble J domain proteins in the ER, Jem1p and Scj1p, to facilitate the export of ERAD substrates to the cytosol by preventing their aggregation in the ER lumen.

Materials and Methods

Yeast Strains and Culture Conditions

Yeast strains used in this study were constructed from SEY6210 (*MAT α ura3 leu2 trp1 his3 lys2 suc2*) (a gift from S. Emr, University of California,

San Diego, San Diego, CA; Robinson et al., 1988), MS10 (*MAT α ura3 leu2 ade2*; a gift from M. Rose (Princeton University, Princeton, NJ), RSY579 (*MAT α kar2-159 ura3 leu2 trp1 ade2*) (a gift from R. Schekman, University of California, Berkeley, Berkeley, CA; Sanders et al., 1992), RDM15-5B (*MAT α sec61-2 ura3 leu2 ade2 pep4*) (a gift from R. Schekman; Deshaies and Schekman, 1987), JN516 (*MAT α SSA1 ssa2-1 ssa3-1 ssa4-1 his3 leu2 ura3 trp1 lys2*), or a1-45 Δ u (*MAT α ssa1-45 ssa2-1 ssa3-1 ssa4-1 his3 leu2 ura3 trp1 lys2*) (Becker et al., 1996). SNY1084-1D (*MAT α ura3 leu2 ade2*) was constructed by crossing MS10 and RSY579. To construct the *prc1-1* mutant, a 3.7-kb EcoRI-SalI fragment of pTSY1000 (a gift from T.M. Stevens, University of Oregon, Eugene, OR; Stevens et al., 1986) containing the *PRC1* gene was subcloned into the EcoRI and SalI sites of pJJ244 (Jones and Prakash, 1990). The *prc1-1* mutation was introduced by oligonucleotide-directed mutagenesis (Kunkel et al., 1987) using an oligonucleotide 5'-CCACATCGCTAGGGAATCTAC-3'. The *sec63-1* and *sec61-2* mutant genes were cloned by PCR using the genomic DNA of RSY151 (*MAT α sec63-1 ura3 leu2 pep4*) (a gift from R. Schekman; Rothblatt et al., 1989) and RDM15-5B as a template, respectively. The amplified DNA fragments were introduced into the polylinker site of pRS306 (Sikorski and Hieter, 1989). The resulting plasmids were linearized and used for introduction of the *prc1-1*, the *sec63-1*, or the *sec61-2* allele by the two step gene replacement method (Rose et al., 1990). Introduction of the mutant alleles into the yeast genome was confirmed by DNA sequencing. The Δ *jem1::LEU2* and Δ *scj1::TRP1* alleles were constructed as described previously (Nishikawa and Endo, 1997). The Δ *scj1::URA3* gene was constructed by replacing the 0.84-kb MunI-XbaI fragment of pBS-SCJ1 (Nishikawa and Endo, 1997) with the 1.2-kb HindIII fragment of pJJ242 containing the *URA3* gene. The *LHS1* gene was cloned by PCR using primers 5'-GCGCTCGAGCGCCATTGAATTGAAC-3' and 5'-GCGGAGCTCGCCAAAGGAGACAGGTGG-3', and the amplified DNA fragment was introduced into the XhoI and SacI sites of pBluescriptII SK+ to give pBSLHS1. The Δ *lhs1::TRP1* allele was constructed by replacing the 2.5-kb EcoRV fragment of pBSLHS1 with the 0.85-kb SmaI-StuI fragment of pJJ281 (Jones and Prakash, 1990) containing the *TRP1* gene. The *DOA4* gene was cloned by PCR using primers 5'-CGCGAGCTCCATAAGGGATGAGTGTA-3' and 5'-GCGCTCGAGCAAACCGTAGACGGCG-3', and the amplified DNA fragment was introduced into the XhoI and the SacI sites of pBluescriptII SK+ to give pBSDOA4. The Δ *doa4::LEU2* allele was constructed by replacing the 1-kb BglII-SalI fragment of pBSDOA4 (Papa and Hochstrasser, 1993) with the 2-kb BamHI-SalI fragment of pJJ282 (Jones and Prakash, 1990) containing the *LEU2* gene. A 3.5-kb DNA fragment containing the Δ *erg6::LEU2* gene was amplified from the genomic DNA of KUY161 (*MAT α erg6::LEU2 ura3 lys2 ade2 his3 leu2*) (a gift from K. Umabayashi, RIKEN, Wako, Japan) by PCR using primers 5'-AATCAACCAAACAGCTAGGGCTGG-3' and 5'-AAGGCCTGCTAGCAATGAACGTGC-3'. Null alleles were introduced into yeast cells by the one step gene disruption method (Rose et al., 1990). Multiple mutants were generated by a second round of transformation. The constructed strains were: KYSC1 (*MAT α prc1-1 ura3 leu2 trp1 his3 lys2 suc2*), KYSC2 (*MAT α prc1-1 Δ jem1::LEU2 ura3 leu2 trp1 his3 lys2 suc2*), KYSC3 (*MAT α prc1-1 Δ scj1::TRP1 ura3 leu2 trp1 his3 lys2 suc2*), KYSC4 (*MAT α prc1-1 Δ jem1::LEU2 Δ scj1::TRP1 ura3 leu2 trp1 his3 lys2 suc2*), KYSC5 (*MAT α prc1-1 sec63-1 ura3 leu2 trp1 his3 lys2 suc2*), KYSC7 (*MAT α sec63-1 ura3 leu2 trp1 his3 lys2 suc2*), KYSC11 (*MAT α sec61-2 Δ jem1::LEU2 ura3 leu2 ade2 pep4*), KYSC13 (*MAT α prc1-1 kar2-159 ura3 leu2 trp1 ade2*), KYSC14 (*MAT α prc1-1 Δ lhs1::TRP1 ura3 leu2 trp1 his3 lys2 suc2*), SNY1026-7A (*MAT α Δ jem1::LEU2 Δ scj1::TRP1 ura3 leu2 trp1 his3 lys2 suc2*), SNY1070 (*MAT α sec61-2 Δ scj1::URA3 ura3 leu2 ade2 pep4*), SNY1071 (*MAT α sec61-2 Δ jem1::LEU2 Δ scj1::URA3 ura3 leu2 ade2 pep4*), SNY1077 (*MAT α sec61-2 SSA1 ssa2-1 ssa3-1 ssa4-1 his3 leu2 ura3 trp1 lys2*), SNY1078 (*MAT α sec61-2 ssa1-45 ssa2-1 ssa3-1 ssa4-1 his3 leu2 ura3 trp1 lys2*), SNY1081 (*MAT α erg6::LEU2 sec61-2 ura3 leu2 ade2 pep4*), SNY1082 (*MAT α doa4::LEU2 sec61-2 ura3 leu2 ade2 pep4*), and SNY1083 (*MAT α prc1-1 ura3 leu2 ade2*). BJ3505 (*MAT α pep4::HIS3 prb1 lys2 trp1 ura3 gal2 can1*) (a gift from Y. Onya, University of Tokyo, Tokyo, Japan) was used for cytosol preparation. Cells were grown in YPD medium containing 1% yeast extract, 2% polypeptone, and 2% glucose. A sulfate-free synthetic minimal medium (Rothblatt and Schekman, 1989) was used for metabolic labeling of yeast cells.

Pulse-Chase Experiments

Metabolic labeling of yeast cells with Tran³⁵S-label (ICN Biomedicals) and preparation of cell extracts were performed as described previously (Nishikawa and Nakano, 1991). When indicated, carbobenzoxy-leuciny-leuci-

nyl-leucinal (MG132; Calbiochem) was added to the labeling medium at 200 μ M for 60 min before the labeling. For the pulse–chase analysis of p α F, cells were pretreated with tunicamycin (10 μ g/ml) for 20 min at 23°C and pulse labeled in the presence of tunicamycin. Immunoprecipitation with anti-CPY or anti–prepro- α -factor (pp α F) antiserum (gifts from A Nakano, RIKEN, Wako, Japan; Jones, 1991) was performed as described by Nishikawa et al. (1994). In the case of immunoprecipitation using an anti-Sec61p antiserum, cell extracts were prepared by incubating at 37°C for 30 min instead of 100°C, and 2.5 μ l of anti-Sec61p antiserum (Stirling et al., 1992) was added to the cell extracts derived from 0.5 OD₆₀₀ cells.

Sucrose Density Gradient Centrifugation

60 OD₆₀₀ units of cells were collected by centrifugation and washed once with ice cold 10 mM Na₂S₂O₃. Cells were suspended in 600 μ l of 20 mM Hepes-KOH, pH 7.4, 50 mM potassium acetate, 2 mM EDTA, 1 mM PMSF, 5 μ g/ml pepstatin A, 2 μ g/ml chymostatin, 5 μ g/ml E-62, 100 μ g/ml *N*-tosyl-L-phenylmethylchloromethyl ketone, 200 μ g/ml *p*-aminobenzamide hydrochloride, 1 mM ϵ -amino capronic acid, 2 μ g/ml aprotinin, 2 μ g/ml antipain, 2 μ g/ml leupeptin, and 20 μ g/ml 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride and disrupted by agitation with glass beads. After removing cell debris by centrifugation, 1% Triton X-100 was added to the cell lysate, which was subsequently centrifuged at 12,000 *g* for 10 min at 4°C. The cleared lysate was layered onto linear sucrose gradients (4 ml, 5–40% sucrose in 20 mM Hepes-KOH, pH 7.4, 50 mM potassium acetate, 2 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, 5 μ g/ml pepstatin A, 2 μ g/ml chymostatin, 5 μ g/ml E-62, 100 μ g/ml *N*-tosyl-L-phenylmethylchloromethyl ketone, 200 μ g/ml *p*-aminobenzamide hydrochloride, 1 mM ϵ -amino capronic acid, 2 μ g/ml aprotinin, 2 μ g/ml antipain, 2 μ g/ml leupeptin, and 20 μ g/ml 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride), and centrifuged at 145,000 *g* for 20 h at 4°C. After centrifugation, fractions were collected from the bottom and analyzed by immunoblotting using anti-CPY antiserum.

In Vitro Translocation and ERAD Assays

Preparations of the yeast microsomes and cytosol fractions were performed as described previously (McCracken and Brodsky, 1996). ³⁵S-labeled pp α F and Δ Gpp α F were prepared in vitro as described previously, and purified over a Sephadex G-25 column (McCracken and Brodsky, 1996; Werner et al., 1996). ER-derived microsomes were incubated with ³⁵S-labeled pp α F or Δ Gpp α F in the presence of an ATP-regenerating system. Microsomes containing Gp α F and Δ Gp α F were collected by centrifugation, washed once, and resuspended in buffer containing an ATP-regenerating system. Microsomes were preincubated at 23 or 37°C for the indicated duration in the absence of the cytosol, but in the presence of ATP. For the analysis of Δ Gp α F degradation, preincubated microsomes were further incubated in the presence of an ATP-regenerating system and cytosol at 37°C. At the indicated time points, proteins were precipitated with trichloroacetic acid and subjected to SDS-PAGE with 18% polyacrylamide gel containing SDS and 4 M urea. For the analysis by sucrose density gradient centrifugation, proteins were extracted from the preincubated suspension by 1% Triton X-100, and after centrifugation at 12,000 *g* for 10 min at 4°C, cleared extracts were subjected to sucrose density gradient centrifugation as described above. After centrifugation, fractions were collected from the bottom and analyzed by SDS-PAGE after trichloroacetic acid precipitation.

Results

Jem1p and *Scj1p*, but Not *Sec63p*, Cooperate with BiP in the ERAD of CPY*

Several lines of evidence show that *Jem1p*, *Scj1p*, and *Sec63p* interact with BiP through their J domains (Brodsky and Schekman, 1993; Scidmore et al., 1993; Schlenstedt et al., 1995; Corsi and Schekman, 1997; Nishikawa and Endo, 1997). To reveal which, if any, of these J domain-containing proteins are involved in ERAD, we tested the effects of functional defects in these proteins on the degradation of CPY*. Although CPY is a vacuole-targeted protein, CPY* does not reach the vacuole but, instead, is

exported from the ER to the cytoplasm and rapidly degraded by the proteasome (Finger et al., 1993; Hiller et al., 1996). When we examined the fate of CPY* in a wild-type strain by pulse–chase analysis, CPY* was found to be degraded with half-lives of 43 min at 23°C, and 12 min at 37°C (Fig. 1, A and B, filled squares). The half-life of CPY* increased 2.7-fold at 23°C in temperature-sensitive BiP (*kar2-159*) mutant cells (Fig. 1 C), suggesting BiP-dependent export of luminal CPY* to the cytosol, as observed previously (Plempner et al., 1997). This defect was exacerbated when *kar2-159* mutant cells were metabolically labeled at 23°C for 10 min and subsequently chased at 37°C. In contrast, cells containing a disruption of the *LHS1* gene degraded CPY* at similar rates to those in wild-type cells at 23 and 37°C (Fig. 1 D). To confirm this result, we measured the degradation of Δ Gp α F, another soluble ERAD substrate (McCracken and Brodsky, 1996) in microsomes prepared from wild-type or the *LHS1*-deleted strain and found that ERAD was unaffected (data not shown). These results indicate that BiP, but not Lhs1p, is involved in the degradation of CPY* in the ER lumen.

Disruption of one of the two genes for the ER luminal DnaJ-like proteins, *Jem1p* and *Scj1p*, did not lead to profound stabilization of CPY* at 23 or 37°C. However, simultaneous disruption of the genes encoding *Jem1p* and *Scj1p* stabilized CPY* significantly, so that the half-life of CPY* increased 2.6-fold at 23°C, and only ~10% of the CPY* was degraded over a 120-min chase at 37°C (Fig. 1, A and B, open circles). Because a precursor form of CPY* was not observed, translocation of CPY* was not delayed in the Δ *Jem1* Δ *Scj1* mutant cells in these experiments (data not shown). To reexamine the role of *Sec63p* in ERAD, we measured the stability of CPY* in *sec63-1* mutant cells (Fig. 1 E). The *sec63-1* mutant strain exhibited a moderate defect in protein import into the ER, even at 23°C, because a precursor form of CPY* (prepro-CPY*) transiently accumulated at early time points during the chase. However, the level of CPY* in the ER lumen (p1 form) subsequently declined with half-lives of 38 min at 23°C and 17 min at 37°C. These half-lives of luminal CPY* do not differ significantly from those in wild-type cells (see above). Thus, the 1.5-fold retardation in the rate of CPY* proteolysis in the *sec63-1* strain that was previously reported (Plempner et al., 1997) was not apparent in these experiments, perhaps because the genetic backgrounds of the strains used in these studies differ. From these data, we conclude that two of the three ER DnaJ-like proteins, *Jem1p* and *Scj1p*, are involved in the ERAD of CPY*.

Defects in BiP or *Jem1p* and *Scj1p* Cause Aggregation of CPY* in the ER

It has been established that Hsp70 binds to and dissociates from unfolded proteins concomitant with ATP binding and hydrolysis, and thereby prevents substrate proteins from aggregating; J domain-containing proteins impact upon this cycle by stimulating ATP hydrolysis (Liberek et al., 1991). The aggregation or formation of high molecular weight oligomers of ERAD substrates in the lumen may hinder retrotranslocation to the cytoplasm. Therefore, we decided to test if defects in the functions of BiP, *Jem1p*, and *Scj1p* promote the aggregation of CPY* when ERAD

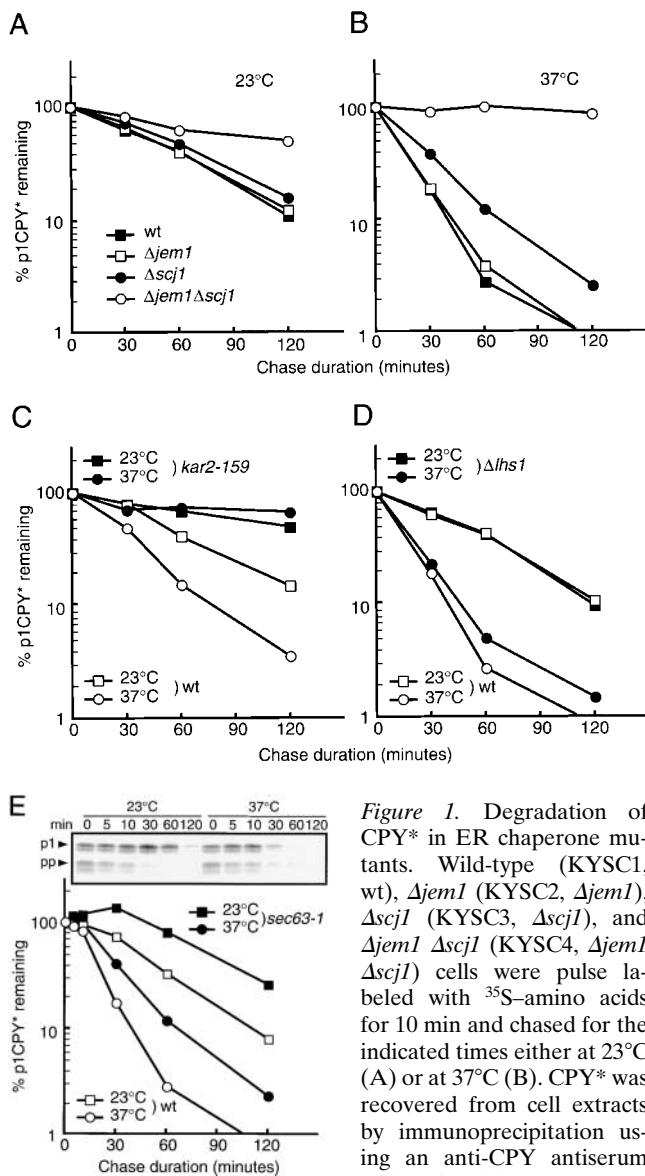


Figure 1. Degradation of CPY* in ER chaperone mutants. Wild-type (KYSC1, wt), $\Delta jem1$ (KYSC2, $\Delta jem1$), $\Delta scj1$ (KYSC3, $\Delta scj1$), and $\Delta jem1 \Delta scj1$ (KYSC4, $\Delta jem1 \Delta scj1$) cells were pulse labeled with ^{35}S -amino acids for 10 min and chased for the indicated times either at 23°C (A) or at 37°C (B). CPY* was recovered from cell extracts by immunoprecipitation using an anti-CPY antiserum and analyzed by SDS-PAGE.

Relative amounts of CPY* were quantified by radioimaging, and the means of two independent experiments are shown. The amount of CPY* at 0 min chase was set to 100%. ■, wild-type; □, $\Delta jem1$; ●, $\Delta scj1$; ○, $\Delta jem1 \Delta scj1$. (C) *kar2-159* (KYSC13, *kar2-159*) and wild-type (SNY1083, wt) cells were pulse labeled with ^{35}S -amino acids for 10 min at 23°C and chased for the times indicated at 23°C or at 37°C. Degradation of CPY* was followed as in A and B. ■, *kar2-159* chased at 23°C; ●, *kar2-159* chased at 37°C; □, wild-type chased at 23°C; ○, wild-type chased at 37°C. (D) $\Delta lhs1$ (KYSC14) cells and wild-type (KYSC1, wt) cells were pulse labeled with ^{35}S -amino acids for 10 min and chased for the times indicated either at 23 or 37°C. Degradation of CPY* was followed as in A and B. ■, $\Delta lhs1$ + at 23°C; ●, $\Delta lhs1$ at 37°C; □, wild-type at 23°C; ○, wild-type at 37°C. (E) *sec63-1* (KYSC5, *sec63-1*) and wild-type (KYSC1, wt) cells were pulse labeled with ^{35}S -amino acids for 10 min at 23°C and chased for the indicated times at 23 or 37°C. Degradation of CPY* was followed as in A and B. ■, *sec63-1* chased at 23°C; ●, *sec63-1* chased at 37°C; □, wild-type at 23°C; ○, wild-type at 37°C; pp, prepro-CPY*; p1, p1CPY*.

is hindered. Wild-type, $\Delta jem1 \Delta scj1$, and *kar2-159* cells grown at 23°C were incubated at 37°C for 60 min, and proteins were extracted from the cells with 1% Triton X-100 and subjected to sucrose density gradient centrifugation. After centrifugation, ~50% of CPY* in wild-type cells was recovered in the fractions corresponding to 70–150 kD (fractions 7–10), and only 13% was found in the pellet (Fig. 2). In contrast, in $\Delta jem1 \Delta scj1$ and *kar2-159* cells, the amount of CPY* recovered in fractions 7–10 was reduced, and 30% (in $\Delta jem1 \Delta scj1$ cells) and 60% (in *kar2-159* cells) of CPY* were recovered in the pellets, indicating that CPY* aggregates in these strains at 37°C. The distribution patterns of CPY* by sucrose density gradient centrifugation did not differ significantly at 23°C between wild-type cells and *kar2-159* or $\Delta jem1 \Delta scj1$ cells (data not shown). We suggest that the aggregated CPY* in the $\Delta jem1 \Delta scj1$ and in *kar2* mutants at 37°C gives rise to the ERAD defect observed in Fig. 1.

BiP and Jem1p/Scj1p Facilitate ERAD of $\Delta GpaF$ by Preventing Aggregate Formation in the ER

To confirm these results, we performed in vitro export/degradation assays with microsomes prepared from $\Delta jem1 \Delta scj1$, *kar2-159*, and *sec63-1* mutant cells. Radiolabeled $\Delta GppaF$ was translocated into microsomes prepared from each of the mutant strains and the isogenic wild-type strain. Upon signal sequence cleavage, the resulting product, $\Delta GpaF$, becomes an ERAD substrate after the washed vesicles are incubated in the presence of cytosol and ATP (McCracken and Brodsky, 1996). When this assay was performed using microsomes prepared from the *kar2-159*, *sec63-1*, and $\Delta jem1 \Delta scj1$ mutant strains, efficient degradation of $\Delta GpaF$ was observed at both 23 and 37°C (data not shown). These results demonstrated that ERAD was proficient in the *kar2-159* and $\Delta jem1 \Delta scj1$ microsomes, in contrast to what was observed using CPY* in vivo (Fig. 1).

To explain this discrepancy, we envisaged that the aggregation of $\Delta GpaF$, and subsequent ERAD defect, might not be apparent unless the translocation and ERAD assays were further uncoupled. Thus, $\Delta GpaF$ -loaded microsomes were first preincubated at 37°C for 20 min in the absence of cytosol but in the presence of ATP, and subsequently incubated with both cytosol and ATP. The additional incubation had no effect on ERAD activity in wild-type microsomes (Fig. 3 A, panel a). However, we now observed that the degradation of $\Delta GpaF$ in the *kar2-159* and $\Delta jem1 \Delta scj1$ mutant microsomes was compromised. Only ~20% of $\Delta GpaF$ was degraded after a 60-min chase for both mutant microsomes (Fig. 3 A, panels b and c, filled circles), in contrast to 40–50% of $\Delta GpaF$ degraded in wild-type microsomes and in the absence of the additional incubation (open circles). Only a slight decrease was observed when ERAD efficiency was examined at 37°C (filled circles) versus 4°C (open circles) in microsomes prepared from the *sec63-1* mutant (Fig. 3 A, panel d). We also found that the extent of the ERAD defect in the *kar2-159* and $\Delta scj1 \Delta jem1$ microsomes correlated with the length of the incubation at 37°C (Fig. 3 B). In contrast, ERAD activity was robust in the wild-type and *sec63-1* microsomes during the same time course at 37°C (Fig. 3 B).

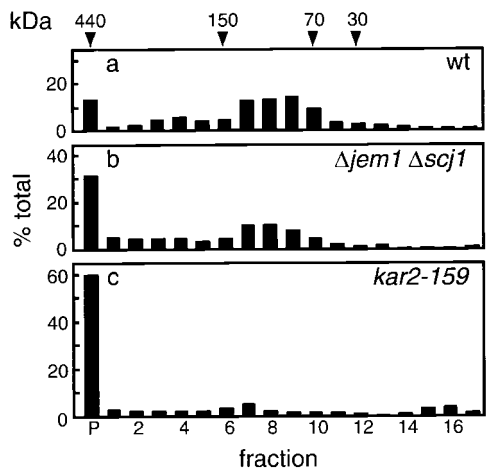


Figure 2. CPY* aggregates in the $\Delta jem1 \Delta scj1$ and the $kar2-159$ mutants. Wild-type (KYSC1, wt), $\Delta jem1 \Delta scj1$ (KYSC4), and $kar2-159$ (KYSC13) cells were grown to early logarithmic phase at 23°C and then shifted to 37°C for 60 min. Cell extracts were prepared and subjected to sucrose density gradient centrifugation as described in the Materials and Methods. Fractions were collected and proteins were analyzed by SDS-PAGE followed by immunoblotting using an anti-CPY antiserum. Relative amounts of CPY* were quantified. Numbers indicate fractions (from bottom to top) and P represents the pellet fraction recovered in the bottom of the tube. Vertical arrowheads show the positions of apoferritin (440 kD), alcohol dehydrogenase (150 kD), bovine serum albumin (70 kD), and carbonic anhydrase (30 kD).

Next, we tested if $\Delta Gp\alpha F$ aggregates in $kar2-159$ and $\Delta jem1 \Delta scj1$ mutant microsomes during the 37°C incubation. $\Delta Gp\alpha F$ and wild-type $pp\alpha F$ were translocated into microsomes prepared from wild-type, $kar2-159$, $\Delta jem1 \Delta scj1$, or $sec63-1$ strains at 20°C, and microsomes were collected by centrifugation and incubated at 23 or 37°C for 20 min in the presence of ATP. Microsomal protein was solubilized with Triton X-100, analyzed by sucrose density gradient centrifugation, and fractions were examined for the presence of radiolabeled protein by SDS-PAGE and radioimaging. At 23°C in all cases, $\Delta Gp\alpha F$ showed a broad range distribution around the fraction corresponding to 70 kD (Fig. 3 C, black bars). When microsomes from $kar2-159$ and $\Delta jem1 \Delta scj1$ cells were incubated at 37°C, the amount of $\Delta Gp\alpha F$ recovered at the bottom of the tube increased significantly. In contrast, the distribution of $\Delta Gp\alpha F$ in the wild-type or $sec63-1$ microsomes incubated at 37°C was essentially unchanged from the results obtained when microsomes had been incubated at 23°C. These data indicate that $\Delta Gp\alpha F$ aggregates at 37°C in $kar2-159$ and $\Delta jem1 \Delta scj1$ microsomes.

When the distribution of wild-type, glycosylated $p\alpha F$ ($Gp\alpha F$) was analyzed under the same conditions, we noted that $Gp\alpha F$ was recovered exclusively in fractions corresponding to proteins of 30–70 kD, regardless of the type of microsomes used and whether they had been incubated at 23 or 37°C (Fig. 3 C, white bars). Therefore, aggregate formation due to defects in the functions of BiP, Jem1p, and Scj1p is specific for an ERAD substrate.

In parallel with these in vitro ERAD assays, we followed the degradation of unglycosylated α -factor in the presence of tunicamycin in vivo (Zhou and Schekman,

1999). We failed to observe a difference in the degradation rates of $\Delta Gp\alpha F$ between wild-type cells and $\Delta jem1 \Delta scj1$ cells at either 23 or 37°C (Fig. 4 A). We suggest that this arises from the fact that the requirement of Scj1p/Jem1p for the degradation of $\Delta Gp\alpha F$ in vitro is most evident only after a further uncoupling of the translocation and ERAD processes (Fig. 3). In vivo, the delivery of $\Delta Gp\alpha F$ from the ER to the cytoplasm and proteasome may be very tightly coupled, obviating the need for these chaperones. In contrast, the degradation of CPY* does require Scj1p/Jem1p (Fig. 1), a phenomenon that may represent the fact that CPY* translocates completely through the pore and into the lumen of the ER before its reexport (Plemper et al., 1999); this may not be the case for $\Delta Gp\alpha F$ in vivo. On the other hand, we observed a partial stabilization of $\Delta Gp\alpha F$ in the $kar2-159$ mutant (Fig. 4 B); after 45 min at 37°C, there was ~34% of the initial material in the $kar2$ mutant strain, compared with ~16% in the wild-type strain. Tunicamycin treatment upregulates the expression of the *KAR2* gene, so that the overproduction of the mutant BiP protein may well interfere with the degradation of $\Delta Gp\alpha F$ by interacting unproductively with $\Delta Gp\alpha F$ or components required for the ERAD process.

Jem1p and Scj1p Do Not Facilitate the ERAD of a Membrane Protein

To determine whether Jem1p and Scj1p are also required for the degradation of an integral membrane ERAD substrate, we examined the fate of a mutant form of Sec61p encoded by the temperature-sensitive *sec61-2* allele. Sec61-2p is selectively degraded by the ubiquitin-proteasome pathway at the restrictive temperature of 38°C, as mutations in ubiquitin-conjugating enzymes or proteasomal subunits inhibit the degradation (Biederer et al., 1996). In agreement with these earlier findings, we found that 56 and 78% of Sec61-2p were degraded after 80 min and 150 min of chase, respectively, in wild-type cells (Fig. 5). We also observed the stabilization of Sec61-2p to a similar extent either when the activity of the ubiquitin-proteasome pathway was compromised by treating cells with MG132 (Fig. 5 A), an inhibitor of the 26S proteasome (Lee and Goldberg, 1996), or when the degradation of Sec61-2p was examined in a strain containing a disruption of the *DOA4* gene (Fig. 5 B), which encodes a deubiquitination enzyme (Papa and Hochstrasser, 1993; Hochstrasser, 1996). On the other hand, we failed to observe significant stabilization of Sec61-2p in strains disrupted for *JEMI*, *SCJ1*, or both genes in combination (Fig. 5 C), compared with the strong stabilization of CPY* and $\Delta Gp\alpha F$ under these conditions (Figs. 1 and 3). It should be noted that the amount of Sec61-2p at 0 min in the chase was higher in the $\Delta scj1$ and the $\Delta jem1 \Delta scj1$ mutant cells because the *SEC61* gene is induced (data not shown). This result is consistent with Scj1p and Jem1p playing a role in ERAD, because conditions that compromise ERAD constitutively upregulate the unfolded protein response in yeast and the *SEC61* gene is one target of this response (Travers et al., 2000).

Because the degradation of three integral ER membrane proteins, Vph1p, CFTR, and Ste6p*, is suppressed in the yeast ER by compromising the activity of the Ssa cy-

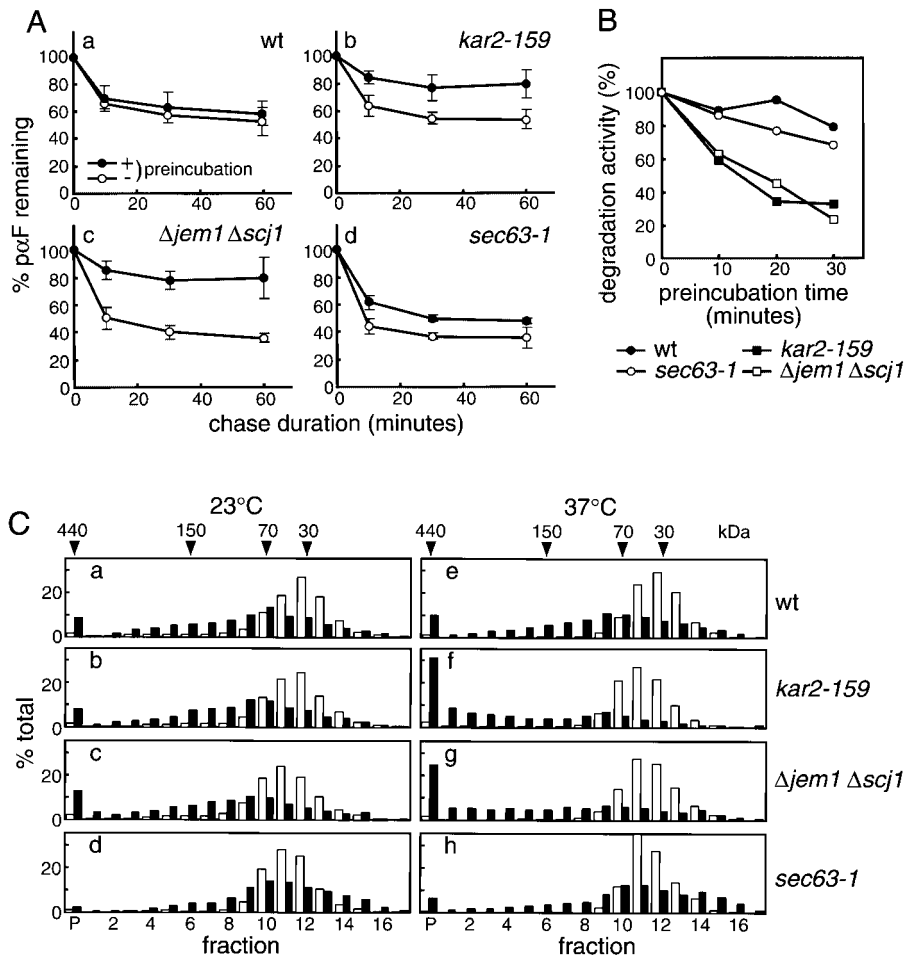


Figure 3. ERAD defects correlate with aggregation of Δ Gpp α F. (A) Radio-labeled Δ Gpp α F was translocated into microsomes prepared from wild-type (a), *kar2-159* (b), Δ *jem1* Δ *scj1* (c), or *sec63-1* (d) strains. After translocation, membranes were incubated on ice (\circ) or at 37°C (\bullet) for 20 min in the presence of ATP. Cytosol prepared from the wild-type strain was added to a final concentration of 5 mg protein/ml, and posttranslocation chase reactions were performed at 37°C for the indicated times. Proteins were precipitated with trichloroacetic acid and analyzed by SDS-PAGE. Relative amounts of Δ Gpp α F were quantified by radioimaging. Error bars indicate standard deviation from the means of at least four independent experiments. The amount of Δ Gpp α F at 0 min chase was set to 100%. (B) Radiolabeled Δ Gpp α F was translocated into microsomes prepared from wild-type (\bullet), *kar2-159* (\blacksquare), Δ *jem1* Δ *scj1* (\square), or *sec63-1* (\circ) strains. Membranes were recovered and incubated at 37°C in the presence of ATP for the indicated times. Cytosol was added to a final concentration of 5 mg protein/ml, and posttranslocation chase reactions were performed at 37°C for 20 min. Degradation activity was represented as the amount of Δ Gpp α F degraded, and the amount of Δ Gpp α F with 0 min preincubation was set to 100%. (C) Radiolabeled Δ Gpp α F and pp α F were

translocated into microsomes prepared from wild-type (a and e), *kar2-159* (b and f), Δ *jem1* Δ *scj1* (c and g), or *sec63-1* (d and h) strains. After translocation, membranes were incubated at 23°C (a–d) or 37°C (e–h) for 20 min in the presence of ATP. Membranes were solubilized with 1% Triton X-100, subjected to sucrose density gradient centrifugation, and analyzed as in the legend to Fig. 2. Relative amounts of Δ Gpp α F and glycosylated pp α F were quantified by radioimaging. Black bars, Δ Gpp α F; white bars, glycosylated pp α F.

tosolic Hsp70 chaperones (Loayza et al., 1998; Hill and Cooper, 2000; Zhang et al., 2001; Harper, C., J.L. Brodsky, and S. Michaelis, manuscript in preparation), we tested whether the proteolysis of Sec61-2p was reduced in the temperature-sensitive *ssal-45* mutant (Becker et al., 1996) at a nonpermissive temperature by pulse-chase analysis. Although the degradation of Vph1p, CFTR, and Ste6p* is

attenuated in this strain under these conditions, the stabilization of Sec61-2p in the *ssal-45* mutant was marginal and comparable to that in the Δ *jem1* Δ *scj1* mutant (Fig. 5 D). An explanation for this discrepancy is provided in the Discussion. Nevertheless, our combined results do indicate that the Jem1 and Scj1 proteins are dispensable for the degradation of Sec61-2p, a conclusion that is in accordance

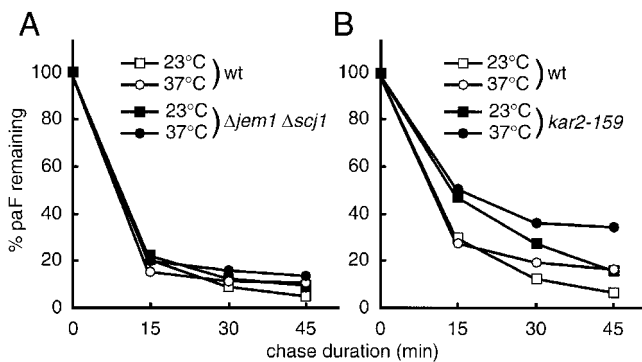


Figure 4. In vivo ERAD analysis of pp α F. (A) Wild-type (SEY6210, wt) and Δ *jem1* Δ *scj1* (SNY1026-7A, Δ *jem1* Δ *scj1*) cells were pulse labeled with 35 S-amino acids at 23°C for 10 min in the presence of 10 μ g/ml tunicamycin, and chased for the indicated times at either 23 or 37°C. pp α F was recovered from cell extracts by immunoprecipitation using an anti-pp α F antiserum and analyzed by SDS-PAGE. Relative amounts of pp α F were quantified by radioimaging and the means of two independent experiments are shown. The amount of pp α F at 0 min chase was set to 100%. \square , wild-type chased at 23°C; \circ , wild-type chased at 37°C; \blacksquare , Δ *jem1* Δ *scj1* chased at 23°C; \bullet , Δ *jem1* Δ *scj1* chased at 37°C. (B) *kar2-159* (RSY579, *kar2-159*) and wild-type (SNY1084-1D, wt) cells were pulse labeled with 35 S-amino acids for 10 min at 23 or 37°C. Degradation of pp α F was followed as in A. \blacksquare , *kar2-159* chased at 23°C; \bullet , *kar2-159* chased at 37°C; \square , wild-type chased at 23°C; \circ , wild-type chased at 37°C.

23°C in the presence of 10 μ g/ml tunicamycin, and were chased for the times indicated at 23 or 37°C. Degradation of pp α F was followed as in A. \blacksquare , *kar2-159* chased at 23°C; \bullet , *kar2-159* chased at 37°C; \square , wild-type chased at 23°C; \circ , wild-type chased at 37°C.

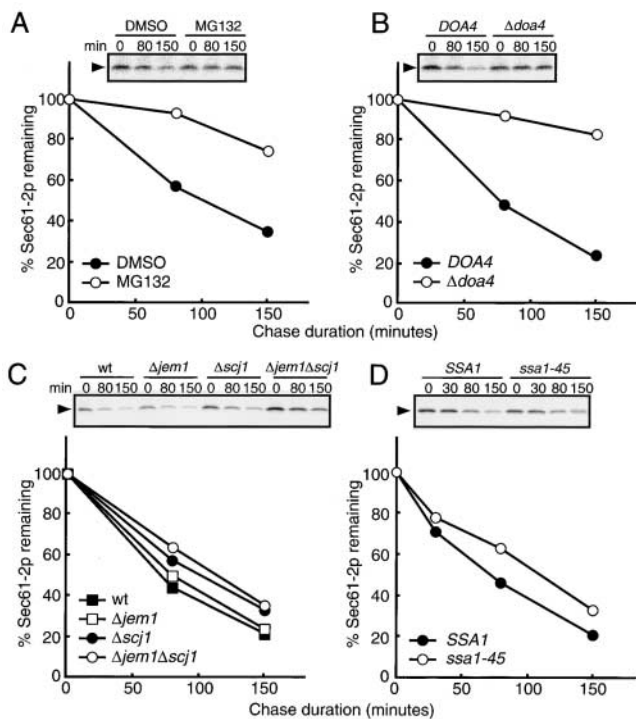


Figure 5. Jem1p and Scj1p are dispensable for the degradation of Sec61-2p. (A) $\Delta erg6$ cells with the *sec61-2* background (SNY1081) were pulse labeled with ^{35}S -amino acids for 10 min, and chased for the times indicated above the lanes at 38°C in the presence (MG132) or the absence (DMSO) of 200 μM MG132. Sec61-2p was recovered from cell extracts by immunoprecipitation using an anti-Sec61p antiserum and analyzed by SDS-PAGE. Relative amounts of Sec61-2p were quantified by radioimaging and the means of two independent experiments are shown. The amount of Sec61-2p at 0 min chase was set to 100%. ●, pulse-chase experiment in the absence of MG132; ○, pulse-chase experiments in the presence of 200 μM MG132. (B) $\Delta doa4$ (SNY1082) and its isogenic wild-type (*DOA4*, RDM15-5B) with the *sec61-2* background were pulse labeled with ^{35}S -amino acids for 10 min, chased for the times indicated above the lanes at 38°C , and analyzed as in A. The means of four independent experiments are shown. The amount of Sec61-2p at 0 min chase was set to 100%. ●, wild-type; ○, $\Delta doa4$. (C) Wild-type (RDM15-5B), $\Delta jem1$ (KYSC11), $\Delta scj1$ (SNY1070), and $\Delta jem1 \Delta scj1$ (SNY1071) cells with the *sec61-2* background were pulse labeled with ^{35}S -amino acids for 10 min, chased for the times indicated above the lanes at 38°C , and analyzed as in A. The means of two independent experiments are shown. The amount of Sec61-2p at 0 min chase was set to 100%. ■, wild-type; □, $\Delta jem1$; ●, $\Delta scj1$; ○, $\Delta jem1 \Delta scj1$. (D) *ssa1-45* (SNY1078) and its isogenic wild-type (*SSA1*, SNY1077) strains with the *sec61-2* background were pulse labeled with ^{35}S -amino acids for 10 min, chased for the times indicated above the lanes at 37°C , and analyzed as in A. The means of two independent experiments are shown. The amount of Sec61-2p at 0 min chase was set to 100%. ●, wild-type; ○, *ssa1-45*.

with previous observations that the ERAD of membrane proteins is BiP independent (Plempner et al., 1998; Brodsky and McCracken, 1999).

Discussion

Misfolded and incorrectly assembled proteins in the ER lumen are distinguished from correctly folded and assembled

proteins, and are degraded by ERAD. We report here that the degradation of CPY* and $\Delta\text{Gp}\alpha\text{F}$, two soluble ERAD substrates, was delayed both in vivo and in vitro when BiP or Scj1p and Jem1p function is attenuated at elevated temperature. These results suggest that Jem1p and Scj1p function as major partners for BiP in the ERAD process. In contrast, ERAD activity appears only marginally affected when Sec63p function is ablated. The role of Sec63p in the ERAD of soluble proteins is controversial (Pilon et al., 1997; Plempner et al., 1997) and may depend on the conditions and assays employed in these unique studies.

To determine the molecular basis for our observations, we developed methods to biochemically analyze the oligomeric state of ERAD substrates. When degradation was compromised either in vivo or in vitro, we noted that the aberrant immature proteins aggregated and migrated in the densest fraction upon sucrose gradient centrifugation. Thus, one role for the BiP-Jem1p-Scj1p chaperone system is to retain ERAD substrates as lower molecular weight species. This is because the diameter of the Sec61p translocation pore has been estimated at 40–60 Å using fluorescent, actively translocating preproteins (Hamman et al., 1998), or 20 Å using three-dimensional reconstruction (Henein et al., 1996); therefore, high molecular weight oligomers or aggregates would remain trapped in the ER lumen and would be prevented from being exported to the cytoplasm for proteasome-mediated degradation. Such a role for molecular chaperones in protein transport through translocation channels is not without precedent. For example, the cytosolic Hsp70s in yeast facilitate protein translocation into both the ER lumen and mitochondria by preventing protein aggregation (Chirico et al., 1988; Deshaies et al., 1988; Murakami et al., 1988; Endo et al., 1996).

We do not rule out the possibility that the action of the BiP-Jem1p-Scj1p system in the quality control of proteins in the ER lumen may be complex, and envision three, non-mutually exclusive scenarios by which these chaperones may engineer ERAD. First, BiP, Jem1p, and Scj1p likely function together to facilitate protein folding and assembly in the ER (Silberstein et al., 1998). Thus, if proteins fail to fold correctly in the ER lumen, BiP may remain bound to them. In wild-type cells, Scj1p/Jem1p could trigger the release of these substrates from BiP, which are then retrotranslocated from the ER and degraded. However, in the absence of Scj1p/Jem1p action, the substrate remains in the ER and eventually aggregates. Second, misfolded proteins may be recognized by components of a secondary ER export machinery. The BiP-Jem1p-Scj1p complex might transfer ERAD substrates to this system, through which they are ultimately delivered to the translocation channel for export. Candidates for this system are the calnexin-calreticulin chaperones that are known to play a role in ERAD (McCracken and Brodsky, 1996; Qu et al., 1996; Liu et al., 1997; for review see Ellgaard et al., 1999). Furthermore, yeast calnexin has been shown to associate both biochemically (Jannatipour et al., 1998) and genetically (Brodsky et al., 1999) with BiP. The future development of an in vitro, ordered, export reaction will address this hypothesis. Third, BiP has been shown to regulate the translocation pore in the mammalian ER (Hamman et al., 1998). It is possible that yeast BiP, in conjunction with Scj1p and Jem1p, performs an analogous role during ret-

rotranslocation. In the event that the channel cannot open, ERAD substrates may aggregate, as observed here.

The Lhs1p chaperone has been shown to aid in the refolding of heat denatured proteins in the ER (Saris et al., 1997). Not surprisingly, an unfolded protein response enhancer resides upstream of the *LHS1* gene, and Lhs1p is induced by conditions that lead to the accumulation of unfolded proteins in the ER (Craven et al., 1996; Cassa-grande et al., 2000; Travers et al., 2000). However, we show here that Lhs1p is not required for ERAD, indicating that its synthesis is induced to help re-fold denatured proteins, but not to destroy them. Thus, the synthetic lethal interactions observed in cells deleted for *LHS1* and containing a thermosensitive *KAR2* allele (Baxter et al., 1996; Craven et al., 1996; Hamilton and Flynn, 1996) suggest that BiP and Lhs1p cooperate in this refolding process.

Our failure to observe SSA-dependent degradation of Sec61-2p (Fig. 5 B) was initially surprising, as three integral membrane ERAD substrates in yeast are stabilized in the *ssa1-45* strain (Hill and Cooper, 2000; Zhang et al., 2001; Harper, C., J.L. Brodsky, and S. Michaelis, manuscript in preparation). However, it should be noted that each of these proteins contains large cytoplasmic domains that may have to be retained in a soluble, degradation-competent conformation. In contrast, Sec61-2p lacks such domains, is overall quite hydrophobic, and therefore its proteolysis might occur independently of cytoplasmic chaperone action. One intriguing hypothesis forwarded by Mayer et al. (1998) is that the proteasome is necessary and sufficient to extract membrane proteins for ERAD. The construction of synthetic, integral membrane ERAD substrates, as was used by these investigators, will allow us to better examine this hypothesis.

Upon sequencing the integrated copy of the *sec61-2* gene used for the experiments described in the legend to Fig. 5, we discovered that the mutation in this thermosensitive allele of *SEC61* alters a glycine to an aspartic acid at position 213 (see Materials and Methods). From the work of Stirling and colleagues (1992), this residue is presumed to lie at the start of transmembrane domain 5 (Wilkinson et al., 1996). This domain contains only 12 amino acids, 11 of which are nonpolar, and has been proposed to require the stabilizing effect of downstream sequences to ensure its proper insertion into the ER membrane. It was also reported that Sec61-2p was stabilized by overexpression of Sss1p, another subunit of the Sec61 complex, and in turn, Sss1p was destabilized in strains containing the *sec61-2* allele (Esnault et al., 1994). The mutation could affect insertion into the ER membrane and/or interactions with Sss1p of Sec61-2p at elevated temperatures. We labeled the *sec61-2* strain at permissive temperature, then switched to restrictive temperature and observed rapid turnover of Sec61-2p (Fig. 4). Therefore, the latter scenario, suggesting the failure of Sec61-2p to interact with Sss1p at elevated temperatures, appears more likely.

In sum, this study reveals one role for the luminal Hsp70 chaperone system in the export of aberrant proteins from the ER lumen to the cytosol, but how ERAD substrates are initially identified and targeted for the export to the cytosol remains unanswered. As exemplified in this work, the continued development of novel biochemi-

cal assays, using genetic tools, will be imperative to continue to define this pathway.

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References

- Baxter, B.K., P. James, T. Evans, and E.A. Craig. 1996. SSI1 encodes a novel Hsp70 of the *Saccharomyces cerevisiae* endoplasmic reticulum. *Mol. Cell Biol.* 16:6444–6456.
- Becker, J., W. Walter, W. Yan, and E.A. Craig. 1996. Functional interaction of cytosolic hsp70 and a DnaJ-related protein, Ydj1p, in protein translocation in vivo. *Mol. Cell Biol.* 16:4378–4386.
- Biederer, T., C. Volkwein, and T. Sommer. 1996. Degradation of subunits of the Sec61p complex, an integral component of the ER membrane, by the ubiquitin-proteasome pathway. *EMBO J.* 15:2069–2076.
- Brizzio, V., W. Khalfan, D. Huddler, C.T. Beh, S.S.L. Andersen, M. Latterich, and M.D. Rose. 1999. Genetic interactions between *KAR7/SEC71*, *KAR8/JEM1*, *KAR5*, and *KAR2* during nuclear fusion in *Saccharomyces cerevisiae*. *Mol. Biol. Cell.* 10:609–626.
- Brodsky, J.L., and A.A. McCracken. 1999. ER protein quality control and proteasome-mediated protein degradation. *Semin. Cell Dev. Biol.* 10:507–513.
- Brodsky, J.L., and R. Schekman. 1993. A Sec63p-BiP complex from yeast is required for protein translocation in a reconstituted proteoliposome. *J. Cell Biol.* 123:1355–1363.
- Brodsky, J.L., E.D. Werner, M.E. Dubas, J.L. Goekeler, K.B. Kruse, and A.A. McCracken. 1999. The requirement for molecular chaperones during endoplasmic reticulum-associated protein degradation demonstrates that protein export and import are mechanistically distinct. *J. Biol. Chem.* 274:3453–3460.
- Cassa-grande, R., P. Stern, M. Diehn, C. Shamu, M. Osario, M. Zuniga, P.O. Brown, and H. Ploegh. 2000. Degradation of proteins from the ER of *S. cerevisiae* requires an intact unfolded protein response pathway. *Mol. Cell.* 5:729–735.
- Chirico, W.J., M.G. Waters, and G. Blobel. 1988. 70K heat shock-related proteins stimulate protein translocation into microsomes. *Nature.* 332:805–810.
- Corsi, A.K., and R. Schekman. 1997. The luminal domain of Sec63p stimulates the ATPase activity of BiP and mediates BiP recruitment to the translocon in *Saccharomyces cerevisiae*. *J. Cell Biol.* 137:1483–1493.
- Craven, R.A., M. Egerton, and C.J. Stirling. 1996. A novel Hsp70 of the yeast ER lumen is required for the efficient translocation of a number of protein precursors. *EMBO J.* 15:2640–2650.
- Deshaies, R.J., and R. Schekman. 1987. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* 105:633–645.
- Deshaies, R.J., B.D. Koch, M. Werner-Washburne, E.A. Craig, and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature.* 332:800–805.
- Ellgaard, L., M. Molinari, and A. Helenius. 1999. Setting the standards: quality control in the secretory pathway. *Science.* 286:1882–1888.
- Endo, T., S. Mitsui, M. Nakai, and D. Roise. 1996. Binding of mitochondrial presequences to yeast cytosolic heat shock protein 70 depends on the amphiphilicity of the presequence. *J. Biol. Chem.* 271:4161–4167.
- Esnault, Y., D. Feldheim, M.-O. Blondel, R. Schekman, and F. Kepes. 1994. SSI1 encodes a stabilizing component of the Sec61 subcomplex of the yeast protein translocation apparatus. *J. Biol. Chem.* 269:27478–27485.
- Finger, A., M. Knop, and D.H. Wolf. 1993. Analysis of two mutated vacuolar proteins reveals a degradative pathway in the endoplasmic reticulum or a related compartment in yeast. *Eur. J. Biochem.* 218:565–574.
- Hamilton, T.G., and G.C. Flynn. 1996. Cer1p, a novel Hsp70-related protein required for posttranslational endoplasmic reticulum translocation in yeast. *J. Biol. Chem.* 271:30610–30613.
- Hamman, B.D., L.M. Hendershot, and A.E. Johnson. 1998. BiP maintains the permeability barrier of the ER membrane by sealing the luminal end of the translocon pore before and early in translocation. *Cell.* 92:747–758.
- Henein, D., K.E.S. Matlack, B. Jungnickel, K. Plath, K.-U. Kalies, K.R. Miller, T.A. Rapoport, and C.W. Akey. 1996. Oligomeric rings of the Sec61p complex induced by ligands required for protein translocation. *Cell.* 87:721–732.
- Hill, K., and A.A. Cooper. 2000. Degradation of unassembled Vph1p reveals novel aspects of the yeast ER quality control system. *EMBO J.* 19:550–561.
- Hiller, M.M., A. Finer, M. Schweiger, and D.H. Wolf. 1996. ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science.* 273:1725–1728.

- Hochstrasser, M. 1996. Ubiquitin-dependent protein degradation. *Annu. Rev. Genet.* 30:405–439.
- Jannatipour, M., M. Callejo, A.J. Parodi, J. Armstrong, and L.A. Rokeach. 1998. Calnexin and BiP interact with acid phosphatase independently of glucose trimming and reglucosylation in *Schizosaccharomyces pombe*. *Biochemistry*. 37:17253–17261.
- Jones, E.W. 1991. Tackling the protease problem in *Saccharomyces cerevisiae*. *Methods Enzymol.* 194:428–453.
- Jones, J.S. and L. Prakash. 1990. Yeast *Saccharomyces cerevisiae* selectable markers in pUC18 polylinkers. *Yeast*. 6:363–366.
- Kelley, W.L. 1998. The J-domain family and the recruitment of chaperone power. *Trends Biochem. Sci.* 23:222–227.
- Kunkel, T.A., J.D. Roberts, and R.A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367–382.
- Lee, D.H., and A.L. Goldberg. 1996. Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 271:22280–22284.
- Liberek, K., J. Marszalek, D. Ang, C. Georgopoulos, and M. Zylicz. 1991. *Escherichia coli* DnaK and GrpE heat shock proteins jointly stimulate ATPase activity of SnaK. *Proc. Natl. Acad. Sci. USA.* 88:2874–2878.
- Liu, Y., P. Choudhury, C.M. Cabral, and R.N. Sifers. 1997. Intracellular disposal of incompletely folded human α 1-antitrypsin involves release from calnexin and post-translational trimming of asparagine-linked oligosaccharides. *J. Biol. Chem.* 272:7946–7951.
- Loayza, D., A. Tam, W.K. Schmidt, and S. Michaelis. 1998. Ste6p mutants defective in exit from the endoplasmic reticulum (ER) reveal aspects of the ER quality control pathway in *Saccharomyces cerevisiae*. *Mol. Biol. Cell.* 9:2767–2784.
- Lyman, S.K., and R. Schekman. 1995. Interaction between BiP and Sec63p is required for the completion of protein translocation into the ER of *Saccharomyces cerevisiae*. *J. Cell Biol.* 131:1163–1171.
- Matlack, K.E., B. Misselwitz, K. Plath, and T.A. Rapoport. 1999. BiP acts as a molecular ratchet during posttranslational transport of prepro- α factor across the ER membrane. *Cell*. 97:553–564.
- Mayer, T.U., T. Braun, and S. Jentsch. 1998. Role of the proteasome in membrane extraction of a short-lived ER transmembrane protein. *EMBO J.* 17:3251–3257.
- McCracken, A.A., and J.L. Brodsky. 1996. Assembly of ER-associated degradation in vitro: dependence on cytosol, calnexin and ATP. *J. Cell Biol.* 132:291–298.
- Murakami, H., D. Pain, and G. Blobel. 1988. 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. *J. Cell Biol.* 107:2051–2057.
- Nishikawa, S., and T. Endo. 1997. The yeast JEM1p is a DnaJ-like protein of the endoplasmic reticulum membrane required for nuclear fusion. *J. Biol. Chem.* 272:12889–12892.
- Nishikawa, S., and A. Nakano. 1991. The GTP-binding Sar1 protein is localized to the early compartment of the yeast secretory pathway. *Biochim. Biophys. Acta.* 1093:135–143.
- Nishikawa, S., A. Hirata, and A. Nakano. 1994. Inhibition of endoplasmic reticulum (ER)-to-Golgi transport induces relocalization of binding protein (BiP) within the ER to form the BiP bodies. *Mol. Biol. Cell.* 5:1129–1143.
- Papa, F.R., and M. Hochstrasser. 1993. The yeast *DOA4* gene encodes a deubiquitinating enzyme related to a product of the human *tre-2* oncogene. *Nature*. 366:313–319.
- Pilon, M., R. Schekman, and K. Römisch. 1997. Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. *EMBO J.* 16:4540–4548.
- Pilon, M., R. Schekman, and K. Römisch. 1998. Sec61p serves multiple roles in secretory precursor binding and translocation into endoplasmic reticulum membrane. *Mol. Biol. Cell.* 9:3455–3473.
- Plempner, R.K., and D.H. Wolf. 1999. Retrograde protein translocation: ERAD-ication of secretory proteins in health and disease. *Trends Biochem. Sci.* 24:266–270.
- Plempner, R.K., S. Bohmler, J. Bordallo, T. Sommer, and D.H. Wolf. 1997. Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature*. 388:891–895.
- Plempner, R.K., R. Egner, K. Kuchler, and D.H. Wolf. 1998. Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdr5 proceeds in a concerted action of Sec61 and the proteasome. *J. Biol. Chem.* 273:32848–32856.
- Plempner, R.K., P.M. Deak, R.T. Otto, and D.H. Wolf. 1999. Re-entering the translocon from the luminal side of the endoplasmic reticulum. Studies on the mutated carboxypeptidase yscY species. *FEBS Lett.* 443:241–245.
- Qu, D., J.H. Teckman, S. Omura, and D.H. Perlmutter. 1996. Degradation of a mutant secretory protein, α 1-antitrypsin Z, in the endoplasmic reticulum requires proteasome activity. *J. Biol. Chem.* 271:22791–22795.
- Robinson, J.S., D.J. Klionsky, L.M. Banta, and S.D. Emr. 1988. Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* 8:4936–4948.
- Römisch, K. 1999. Surfing the Sec61 channel: bidirectional protein translocation across the ER membrane. *J. Cell Sci.* 112:4185–4191.
- Rose, M.D., F. Winston, and P. Hieter. 1990. *Methods in Yeast Genetics: A Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 198 pp.
- Rothblatt, J., and R. Schekman. 1989. A hitchhiker's guide to analysis of the secretory pathway in yeast. *Methods Cell Biol.* 32:3–36.
- Rothblatt, J.A., R.J. Deshaies, S.L. Sanders, G. Daum, and R. Schekman. 1989. Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. *J. Cell Biol.* 109:2641–2652.
- Sadler, I., A. Chiang, T. Kurihara, J. Rothblatt, J. Way, and P. Silver. 1989. A yeast gene important for protein assembly into the endoplasmic reticulum and the nucleus has homology to DnaJ, an *Escherichia coli* heat shock protein. *J. Cell Biol.* 109:2665–2675.
- Sanders, S.L., K.M. Whitfield, J.P. Vogel, M.D. Rose, and R.W. Schekman. 1992. Sec61p and BiP directly facilitate polypeptide translocation into the ER. *Cell.* 69:353–365.
- Saris, N., H. Holkeri, R.A. Craven, C.J. Stirling, and M. Makarow. 1997. The hsp70 homologue Lhs1p is involved in a novel function of the yeast endoplasmic reticulum, refolding and stabilization of heat-denatured protein aggregates. *J. Cell Biol.* 137:813–824.
- Schlenstedt, G., S. Harris, B. Risse, R. Lill, and P.A. Silver. 1995. A yeast DnaJ homologue, Scj1p, can function in the endoplasmic reticulum with BiP/Kar2p via a conserved domain that specifies interactions with Hsp70s. *J. Cell Biol.* 129:979–988.
- Scidmore, M.A., H.H. Okamura, and M.D. Rose. 1993. Genetic interactions between KAR2 and SEC63 encoding homologues of DnaK and DnaJ in the endoplasmic reticulum. *Mol. Biol. Cell.* 4:1145–1159.
- Silberstein, S., G. Schlenstedt, P.A. Silver, and R. Gilmore. 1998. A role for the DnaJ homologue Scj1p in protein folding in the yeast endoplasmic reticulum. *J. Cell Biol.* 143:921–933.
- Sikorski, R.S. and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics.* 122:19–27.
- Simons, J.F., S. Ferro-Novick, M.D. Rose, and A. Helenius. 1995. BiP/Kar2p serves as a molecular chaperone during carboxypeptidase Y in yeast. *J. Cell Biol.* 130:41–49.
- Stevens, T.H., J.H. Rothman, G.S. Payne, and R. Schekman. 1986. Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. *J. Cell Biol.* 102:1551–1557.
- Stirling, T.H., J. Rothblatt, M. Hosobuchi, R. Deshaies, and R. Schekman. 1992. Protein translocation mutants defective in the insertion of integral membrane proteins into the endoplasmic reticulum. *Mol. Biol. Cell* 3:129–142.
- Travers, K.J., C.K. Patil, L. Wodicka, D.J. Lockhart, J.S. Weissman, and P. Walter. 2000. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell.* 101:249–258.
- Werner, E.D., J.L. Brodsky, and A.A. McCracken. 1996. Proteasome-associated endoplasmic reticulum associated protein degradation: an unconventional route to a familiar fate. *Proc. Natl. Acad. Sci. USA.* 93:13797–13801.
- Wiertz, E.J., D. Tortorella, M. Bogoy, J. Yu, W. Mothes, T.R. Jones, T.A. Rapoport, and H.L. Ploegh. 1996. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature*. 384:432–438.
- Wilkinson, B.M., A.J. Critchley, and C.J. Stirling. 1996. Determination of the transmembrane topology of yeast Sec61p, an essential component of the endoplasmic reticulum translocation complex. *J. Biol. Chem.* 271:25590–25597.
- Wilkinson, B.M., J.R. Tyson, P.J. Reid, and C.J. Stirling. 2000. Distinct domains within yeast Sec61p involved in post-translational translocation and protein dislocation. *J. Biol. Chem.* 275:521–529.
- Zhang, Y., G. Nijbroek, M.L. Sullivan, A.A. McCracken, S.C. Watkins, S. Michaelis, and J.L. Brodsky. 2001. The Hsp70 molecular chaperone facilitates the ER associated protein degradation of the cystic fibrosis transmembrane conductance regulator in yeast. *Mol. Biol. Cell.* 12:1303–1314.
- Zhou, M., and R. Schekman. 1999. The engagement of Sec61p in the ER dislocation process. *Mol. Cell.* 4:925–934.