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 lumenal 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 temperatures 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 (Elgaard 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 ΔGapαF, an unglycosylated mutant form of the yeast α mating pheromone pro–α-factor (pαF), 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 α1-protease inhibitor, A1PiZ, was observed in yeast cells containing temperature-sensitive mutations in KAR2, the gene encoding yeast BiP, and the degradation of ΔGapαF was inhibited in microsomes prepared from temperaturesensitive 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
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 (Sandler 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 likely involved in the transport of CPY*. Because the quality control system is inefficient for protein import into the ER, but is defective for ERAD of CPY* and the ERAD of CPY* and BiP-like DnaJ-like proteins in the ER lumen, are involved in facilitating 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 (MATa ura3-52 leu2-3,112 trpl1 his3Δ1 lys2Δ2) (a gift from S. Ener, University of California, San Diego, San Diego, CA; Robinson et al., 1988), MS10 (MATa ura3 leu2 ade2; a gift from M. Rose (Princeton University, Princeton, NJ), RYS57 (MATa kar2-159 his3Δ1 leu2-3 trpl1 ade2) (a gift from R. Schekman; Schekman, University of California, Berkeley, CA; Sanders et al., 1992), RDM5-15B (MATa sec61-2 ura3 leu2 ade2 pep4) (a gift from R. Schekman; Deshaies and Schekman, 1987), JN516 (MATa SSA1 ssd1-1 ssd1-4 his3 leu2 ura3 trpl1 lys2), or ala-145a (MATa ssal-45 ssd1-3 ssd1-4 his3 leu2 ura3 trpl1 lys2) (Becker et al., 1986). SNY1084-1D (MATa ura3 MS43 ade2) was constructed by crossing MS10 (a gift from R. Schekman; Rothblatt et al., 1989) and RDM15-5B as a template, respectively. The amplified DNA fragments were introduced into the polynucleotide site of pRS506 (Sikorski and Hieter, 1989). The resulting plasmids were linearized and used for introduction of the prel-1, 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 Δjem1::LEU2 and Δscj1::TRP1 alleles were constructed as described previously (Nishikawa and Endo, 1997). The Δjem1::URA3 gene was constructed by replacing the 0.84-kb Munl-Xbal fragment of RSY151 (Nishikawa and Endo, 1997) with the 1.2-kb HindIII fragment of pJ242 containing the URA3 gene. The URA3 gene was cloned by PCR using primers 5′-GCGCTCGAGCCATTAATCCAGTTAGGAAC-3′ and 5′-GCGCTCACGTCGCAATAAAGGATGTA-3′, and the amplified DNA fragment was introduced into the XhoI and SacI sites of pBluescriptII (Jones and Prakash, 1990) containing the sec61-2 mutant genes were cloned by PCR using primers 5′-GCCGAGCTCCATAAAGGGATGTA-3′ and 5′-GCCGCTCACGTCGCAATAAAGGATGTA-3′, and the amplified DNA fragment was introduced into the XhoI and SacI sites of pBluescriptII SK+ to give pBSLHS1. The Δhis1::TRP1 allele was constructed by replacing the 2.5-kb EcoRV fragment of pBSLHS1 with the 0.85-kb Smal-SstI fragment of pJ282 (Jones and Prakash, 1990) containing the LEU2 gene. A 3.5-kb DNA fragment containing the Δsac3::LEU2 gene was amplified from the genomic DNA of KUY161 (MATa erg6::LEU2 ura3 lys2 ade2 his3 leu2) (a gift from K. Umebayashi, RIKEN, Wako, Japan) with PCR using primers 5′-AAATCCCAAGCATAGCTAC-3′ and 5′-AAAGTCCTGTAAGCTAGCAT-3′ (a gift from K. Prakash, 1993) with the 2-kb BamHI-Sall fragment of pJ282 (Jones and Prakash, 1990) containing the LEU2 gene. 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 DgopF. CPY* and DgopF aggregate in a temperature-sensitive kar2 mutant and the Δjem1Δscj1 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.

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, carbobenxoxyl-leucinyl-leucinyl-
n-y-l-leucinal (MG132; Calbiochem) was added to the labeling medium at 200 μM for 60 min before the labeling. For the pulse-chase analysis of pof cells, cells were pretreated with tunicamycin (10 μg/ml) for 20 min at 23°C and pulse labeled in the presence of tunicamycin. Immunoprecipitation with anti-CBP or anti-prepro-α-factor (pofF) 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-See61p antiserum, cell extracts were prepared by incubating at 37°C for 30 min in stead of 10°C, and 2.5 μl of anti-See61p 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 NaCl. Cells were suspended in 600 μl of 20 mM Hepes-KOH, pH 7.4, 50 mM potassium acetate, 2 mM EDTA, 1 mM PMSF, 5 μg/ml pepstatin A, 2 μg/ml chymostatin, 5 μg/ml E-62, 100 μg/ml N-tosyl-l-phenylmethylchloromethyl ketone, 200 μg/ml p-aminobenzamidine hydrochloride, 1 mM e-aminocaproic acid, 2 μg/ml aprotinin, 2 μg/ml antipain, 2 μg/ml leupeptin, and 20 μg/ml 4-(2-aminoethyl) benzenesulfonfonyl 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 μg/ml pepstatin A, 2 μg/ml chymostatin, 5 μg/ml E-62, 100 μg/ml N-tosyl-l-phenylmethylchloromethyl ketone, 200 μg/ml p-aminobenzamidine hydrochloride, 1 mM e-aminocaproic acid, 2 μg/ml aprotinin, 2 μg/ml antipain, 2 μg/ml leupeptin, and 20 μg/ml 4-(2-aminoethyl) benzenesulfonfonyl fluoride hydrochloride), and centrifuged at 145,000 g for 2 h at 4°C. After centrifugation, fractions were collected from the bottom and analyzed by immunoblotting using anti-CBP antisera.

**In Vitro Translocation and ERAD Assays**

Preparations of the yeast microsome and cytosol fractions were performed as described previously (McCracken and Brodsky, 1996). 35S-labeled pofF and ΔGppF 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 pofF or ΔGppF in the presence of an ATP-regenerating system. Microsomes containing GppF and ΔGppF 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 ΔGppF 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 37°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 (Plemper 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 ΔGppF, 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 Δjem1Δscj1 mutant cells in these experiments (data not shown). To reexamine the role of Sec63p in ERAD, we measured the stability of CPY* in sec63-I mutant cells (Fig. 1 E). The sec63-I 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-I strain that was previously reported (Plemper 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*.
is hindered. Wild-type, Δjem1Δscj1, 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 Δjem1Δscj1 and kar2-159 cells, the amount of CPY* recovered in fractions 7–10 was reduced, and 30% (in Δjem1Δscj1 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 Δjem1Δscj1 cells (data not shown). We suggest that the aggregated CPY* in the Δjem1Δscj1 and in kar2 mutants at 37°C gives rise to the ERAD defect observed in Fig. 1.

**BiP and Jem1p/Scj1p Facilitate ERAD of ΔGppF by Preventing Aggregate Formation in the ER**

To confirm these results, we performed in vitro export/degradation assays with microsomes prepared from Δjem1Δscj1, kar2-159, and sec63-1 mutant cells. Radiolabeled ΔGppF was translocated into microsomes prepared from each of the mutant strains and the isogenic wild-type strain. Upon signal sequence cleavage, the resulting product, ΔGppF, 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 Δjem1Δscj1 mutant strains, efficient degradation of ΔGppF was observed at both 23 and 37°C (data not shown). These results demonstrated that ERAD was proficient in the kar2-159 and Δjem1Δscj1 microsomes, in contrast to what was observed using CPY* in vivo (Fig. 1).

To explain this discrepancy, we envisaged that the aggregation of ΔGppF, and subsequent ERAD defect, might not be apparent unless the translocation and ERAD assays were further uncoupled. Thus, ΔGppF-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 ΔGppF in the kar2-159 and Δjem1Δscj1 mutant microsomes was compromised. Only ~20% of ΔGppF 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 ΔGppF 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 Δscj1Δjem1 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).
Next, we tested if ΔGpaF aggregates in kar2-159 and Δjem1Δscj1 mutant microsomes during the 37°C incubation. ΔGpaF and wild-type ppaF were translocated into microsomes prepared from wild-type, kar2-159, Δjem1Δscj1, 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, ΔGpaF showed a broad range distribution around the fraction corresponding to 70 kD (Fig. 3 C, black bars). When microsomes from kar2-159 and Δjem1Δscj1 cells were incubated at 37°C, the amount of ΔGpaF recovered at the bottom of the tube increased significantly. In contrast, the distribution of ΔGpaF 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 ΔGpaF aggregates at 37°C in kar2-159 and Δjem1Δscj1 microsomes.

When the distribution of wild-type, glycosylated pαF (GpaF) was analyzed under the same conditions, we noted that GpaF 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 ΔGpaF between wild-type cells and Δjem1Δscj1 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 ΔGpaF in vitro is most evident only after a further uncoupling of the translocation and ERAD processes (Fig. 3). In vivo, the delivery of ΔGpaF 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 ΔGpaF in vivo. On the other hand, we observed a partial stabilization of ΔGpaF 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 ΔGpaF by interacting unproductively with ΔGpaF 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 JEM1, SCI1, or both genes in combination (Fig. 5 C), compared with the strong stabilization of CPY* and ΔGpaF 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 Δscj1 and the Δjem1Δscj1 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 cytochrome c oxidase,
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 ssa1-45 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 ssa1-45 mutant was marginal and comparable to that in the Δjem1 Δscj1 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 with our previous findings that the Jem1 and Scj1 proteins are dispensable for the degradation of Sec61-2p, a conclusion that is in accordance with our previous findings that the Jem1 and Scj1 proteins are dispensable for the degradation of Sec61-2p, a conclusion that is in accordance with our previous findings. The Journal of Cell Biology, Volume 153, 2001 1066
with previous observations that the ERAD of membrane proteins is BiP independent (Plemper 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 ΔGpαF, two soluble ERAD substrates, was delayed both in vivo and in vitro when BiP or Scjlp and Jem1lp function is attenuated at elevated temperature. These results suggest that Jem1lp and Scjlp function as major partners for BiP in the ERAD process. In contrast, ERAD activity appears only marginally affected when Sec63lp function is ablated. The role of Sec63lp in the ERAD of soluble proteins is controversial (Pilon et al., 1997; Plemper 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-Jem1lp-Scjlp chaperone system is to retain ERAD substrates as lower molecular weight species. This is because the diameter of the Sec63lp translocation pore has been estimated at 40–60 Å using fluorescently, 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 cytosol 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-Jem1lp-Scjlp 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, Jem1lp, and Scjlp 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, Scjlp/Jem1lp could trigger the release of these substrates from BiP, which are then retrotranslocated from the ER and degraded. However, in the absence of Scjlp/Jem1lp function, 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-Jem1lp-Scjlp 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 Scjlp and Jem1lp, performs an analogous role during ret-

Figure 5. Jem1lp and Scjlp are dispensable for the degradation of Sec61-2p. (A) Δerg6 cells with the sec61-2 background (SNY1081) were pulse labeled with 35S–amino acids for 10 min, and chased for the times indicated above the lanes at 38°C in the presence of 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 experiment in the presence of 200 μM MG132. (B) Δdoa4 (SNY1082) and its isogenic wild-type (DOA4, RDM15-5B) with the sec61-2 background were pulse labeled with 35S–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; , Δdoa4. (C) Wild-type (RDM15-5B), Δjem1 (KYSC11), Δscj1 (SNY1070), and Δjem1 Δscj1 (SNY1071) cells with the sec61-2 background were pulse labeled with 35S–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; □, Δjem1; , Δscj1; , Δjem1 Δscj1. (D) ssa1-45 (SNY1078) and its isogenic wild-type (SSA1, SNY1077) strains with the sec61-2 background were pulse labeled with 35S–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.
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; Cassagrande 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 ssal-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 biochemical assays, using genetic tools, will be imperative to continue to define this pathway.

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