The Requirement for Molecular Chaperones during Endoplasmic Reticulum-associated Protein Degradation Demonstrates That Protein Export and Import Are Mechanistically Distinct*

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Polypeptide import into the yeast endoplasmic reticulum (ER) requires two hsp70s, Ssa1p in the cytosol and BiP (Kar2p) in the ER lumen. After import, aberrant polypeptides may be exported to the cytoplasm for degradation by the proteasome, and defects in the ER chaperone calnexin (Cne1p) compromise their degradation. Both import and export require BiP and the Sec61p translocation complex, suggesting that import and export may be mechanistically related. We now show that the cne1Δ and two kar2 mutant alleles exhibit a synthetic interaction and that the export and degradation of pro-α factor is defective in kar2 mutant microsomes. Pulse-chase analysis indicates that A1PIZ, another substrate for degradation, is stabilized in the kar2 strains at the restrictive temperature. Because two of the kar2 mutants examined are proficient for polypeptide import, the roles of BiP during ER protein export and import differ, indicating that these processes must be mechanistically distinct. To examine whether Ssa1p drives polypeptides from the ER and is also required for degradation, we assembled reactions using strains either containing a mutation in SSA1 or in which the level of Ssa1p could be regulated. We found that pro-α factor and A1PIZ were degraded normally, indicating further that import and export are distinct and that other cytotoxic factors may pull polypeptides from the ER.

Results from recent studies have established the existence of an intracellular protein degradation process that removes aberrant and unassembled proteins from the endoplasmic reticulum (ER) (reviewed in Refs. 1–3). ER-associated protein degradation (ERAD) operates as an essential step in the quality control of newly synthesized proteins by selectively degrading aberrant and unassembled secretory proteins that accumulate within the ER. It is now clear that the degradation of many soluble (4, 5) and integral membrane (6–17) proteins in the ER involves both the ER and the cytoplasmic proteasome, indicating that the energy source required to drive these proteins from the ER, however, remains unknown, but it has been proposed to derive from the action of cytosolic molecular chaperones (1).

Several studies suggest that the protein translocation channel, composed of the Sec61p complex, mediates polypeptide extrusion from the ER. First, CPY*, a mutated form of vacuole-targeted carboxypeptidase Y, was stabilized in yeast strains containing a mutation in SEC61 (18). Second, the degradation of an unglycosylated form of the yeast mating pheromone, pro-α factor (pαF), was inhibited in microsomes prepared from sec61 mutant strains (19). Third, ERAD substrates were co-immunoprecipitated with the mammalian Sec61p homologue en route to degradation (9, 17).

A vital aspect of ERAD is its selectivity for specific soluble and integral membrane proteins, whereas the majority of ER resident and secreted proteins are stable (20, 21). Although the nature of this substrate selectivity is not completely clear, structural motifs that are buried when proteins are correctly folded and assembled may be recognized by the ERAD machinery if protein folding is hindered (22–24). Two ER lumenal molecular chaperones, BiP and calnexin, are known to be involved in protein maturation and “quality control” in the ER and are likely candidates to play a role in ERAD.

Calnexin is a lectin that binds transiently to monoglucosylated glycans and retains unfolded polypeptides in the ER lumen (25, 26); therefore, calnexin could target misfolded proteins for ERAD. Support for this hypothesis derives from the observation that some mutant proteins in the mammalian ER bind to calnexin prior to their degradation (12, 27, 28) and that partially inhibiting the interaction between calnexin and ApoB attenuated the ER-associated degradation of ApoB ~2-fold (29). In addition, we discovered that ER-derived microsomes prepared from a yeast strain deleted for calnexin were compromised in their ability to degrade pαF in vitro (30).

BiP, an hsp70 homologue, binds preferentially to hydrophobic arrays of amino acids (31, 32), suggesting that BiP can recognize unfolded proteins. BiP is also required for protein translocation into the ER lumen in yeast, acting as a ratchet to prevent polypeptide egress from the ER, as a motor to actively drive substrates into the ER, and/or in conjunction with other proteins to regulate the translocation machinery (reviewed in Ref. 33). Thus, BiP might promote polypeptide efflux during ERAD.

Several investigators have uncovered a role for BiP during ERAD. First, iodination of a misfolded proinsulin polypeptide in vivo indicated co-localized signals for BiP binding and ERAD (24). Second, the dissociation of BiP from unassembled Ig chains correlated with the kinetics of Ig degradation (34). Third, Skowronek et al. (35) recently determined that the ac-
celerated degradation of immunoglobulin light chain chimeras corresponded to enhanced BiP binding. And fourth, Plemer et al. (18) observed the inefficient degradation of CPY* in yeast containing a temperature-sensitive mutation in the gene encoding BiP, KAR2.

Because the kar2 mutant used by Plemer et al. (18) to examine ERAD in yeast is also defective for protein translocation into the ER (36, 37), BiP may have been performing the same function during polypeptide export as in import. Therefore, to determine whether the function of BiP during polypeptide export and degradation is more elaborate than its role during translocation, we measured ERAD both in vitro and in vivo using kar2 mutants that are translocation-proficient, as well as the kar2 mutant used previously (18). Our data demonstrate that each of these mutants is defective for ERAD, indicating for the first time that BiP action during protein translocation into the ER and polypeptide efflux from the ER differs.

Another hs70 molecular chaperone, Ssa1p, is a cytosolic protein known to function in polypeptide import into the yeast ER (38, 39). To investigate whether Ssa1p was required to drive ERAD substrates from the ER, we examined ERAD in vitro and in vivo using strains either containing a temperature-sensitive mutation in SSA1 (38) or in which the levels of Ssa1p can be regulated (39, 40); however, we observed that ERAD activity was unaffected. We conclude from these combined results that BiP and Ssa1p play distinct roles during protein import and export and that the molecular mechanisms of polypeptide import and export from the yeast ER must differ.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—RSY607 (MATa leu2-3, 112 ura3-52 his3-11, 15 leu2-3, 112 trp1-1 ade2-101 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 cne1-1:LEU2) (41); MS10 (MATa ade2-101 leu2-3, 112 ura3-52 kar2-113), MS111 (MATa ade2-101 leu2-3, 112 ura3-52 kar2-1), and MS193 (MATa ade2-101 leu2-3, 112 ura3-52 kar2-133) (36, 37, 42, 43); MW141 (MATa his3-11, 15 leu2-3, 112 trp1-1 ura3-52 ssa1-1:HIS3 ssa2-2:LEU2 ssa3-2:URA3 pGAL-SAL1-TRP1) (39); JN516 (MATa his3-11, 15 leu2-3, 112 lys2-52 trp1-1 ssa1-1 ssa2-1 ssa3-1 sus2-2), and a1-45AU (MATa his3-11, 15 leu2-3, 112 lys2-52 trp1-1 ssa1-1 ssa2-1 ssa3-2 ssa4-2) (38); cells containing ssa1-45BKD ssa2-1 ssa3-2 ssa4-2 are temperature-sensitive for growth and protein translocation (38). Strains were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) at 30°C unless otherwise indicated.

**Yeast Strains and Growth Conditions**—Strains were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) at 30°C unless otherwise indicated.

**Luciferase Assay**—The ability of an hspl0 chaperone to retain heat-denatured luciferase in a folding-competent conformation was assayed as described (47) with minor modifications. Briefly, firefly luciferase was incubated in the presence of the yeast hspl0 chaperone, Sse1p, at a 1:5 molar ratio and the mixture was incubated at 42°C for 30 min. An ATP-regenerating system (37) and yeast cytosol from the desired strain was added to a final concentration of 4 mg/ml, and the reaction was incubated at 26°C. Aliquots at the indicated time points were assayed for luciferase activity in an Analytical Luminescence Laboratory (Ann Arbor, MI) Monolight luminometer using luciferin as the substrate. Although the refolding of luciferase in this assay requires cytosol, purified cytosolic hspl0 and a cognate DnaJ chaperone can substitute for the cytosolic requirement (47), indicating that the chaperone activity of an hspl0 may be assayed using this procedure. Sse1p was purified from yeast over-expressing a hexa-histidine tagged form of Sse1p using nickel agarose affinity and ion exchange chromatography, and yeast cytosol was prepared as described previously (30).

**RESULTS**

**The Genes Encoding BiP and Calnexin Interact**—We previously observed that ERAD was inhibited by ~50% when assayed in yeast microsomes prepared from a strain deleted for calnexin (cne1) (30). Multichaperone complexes containing BiP and calnexin have been noted in the mammalian ER (48), and BiP and calnexin co-immunoprecipitate in extracts prepared from the yeast *Schizosaccharomyces pombe*. Therefore, we sought genetic interactions between the cne1 mutation and mutations in BiP (kar2–1 and kar2–133) that may prevent the ability of BiP to chaperone protein folding in the ER (43, 49). To this end, we mated the cne1 deleted strain (41) to kar2–1 and kar2–133 and dissected ascii after sporulation of the diploids. Tetratype tetrads with respect to CNE1 and KAR2 were then identified. At least two representative tetratypes from each cross were analyzed, replicated, and grown at 30, 35, or 37°C. As shown in Fig. 1, A and B, we observed that the kar2–1cne1Δ and kar2–133cne1Δ strains grew less well than the corresponding kar2–1 and kar2–133 strains at the semipermissive temperature of 35°C, indicating a mild synthetic interaction between the kar2 and the cne1 mutations. To confirm this result, we performed growth curves at 35°C of each strain obtained from the kar2 × cne1Δ crosses (Fig. 1, C and D). We noted again that the kar2 mutants grew somewhat better than the kar2cne1Δ double mutant strains at this temperature. These results suggest that BiP and calnexin may act in a common pathway in *S. cerevisiae*.

*BiP Is Required for ERAD in Vitro and in Vivo*—To examine
leucine in the ATPase domain of BiP (42), obstructs the import of polypeptides at 37 °C by inhibiting the association of translocating proteins with Sec61p (36), and compromises the degradation of an ERAD substrate, CPY*, \textit{in vivo} (18). Interestingly, Kar2–113p exhibits wild type ATPase activity but reduces the levels of both co- and posttranslational protein translocation into ER-derived microsomes (37). These results, and others, suggest that BiP may regulate the translocation machinery (33), a function likely to hinder ERAD because degraded substrates must be exported from the ER through the Sec61p complex (9, 18, 19). By comparison, the kar2–1 and kar2–133 mutations are in the peptide binding domain of BiP (kar2–1, P515L; kar2–133, T473F) (42), and protein translocation is unaffected, as determined by examining whether two ER-targeted substrates (ppaF and preinvertase) accumulate \textit{in vivo} at the nonpermissive temperature (42) and by examining whether the posttranslational translocation of an ER-targeted substrate (ppaF) (37) was compromised \textit{in vitro} (see below); however, these mutations may affect the folding of specific proteins in the yeast ER (43, 49). We anticipated that defects in the ability of BiP to chaperone protein folding may also obstruct ERAD.

To test this hypothesis, we translocated radiolabeled ΔGpppF into microsomes prepared from each mutant strain and an isogenic wild type strain. ΔGpppF is a form of the yeast mating pheromone precursor, ppaF, that cannot become glycosylated after signal sequence cleavage. The resulting product, ppaF, is a substrate for ERAD \textit{in vitro} when a posttranslocation chase is performed in the presence of wild type cytosol and hydrolyzable ATP (5, 19, 30). We found that the extent of ΔGpppF translocated into kar2–1 and kar2–133 mutant microsomes and wild type microsomes was similar, whereas translocation in the kar2–113 microsomes was ~52% as efficient as in wild type microsomes (data not shown), in accordance with previous data (36, 37, 42). In a subsequent ERAD assay, we observed that the degradation of ppaF was slower in each of the mutants at both permissive (23 °C) and nonpermissive (37 °C) temperatures and that the defect was more pronounced at the nonpermissive temperature (Fig. 2). At 23 °C, ~64% of ppaF was degraded in wild type microsomes during the 40-min chase incubation, whereas only 45, 34, and 45% was degraded in microsomes derived from kar2–1, kar2–113, and kar2–133, respectively, at the 40-min time point. At 37 °C, whereas 64% of ppaF was degraded in wild type microsomes, only 36, 22, and 41% was degraded in microsomes derived from kar2–1, kar2–113, and kar2–133, respectively. These results suggest that Kar2p may be a component of the recognition and/or export machinery required for the degradation of ppaF, as was suggested previously for CPY* (18).

To determine whether the mutations in KAR2 prevented ppaF export from the ER-derived microsomes, a protease protection analysis was performed (5). In this assay, exported ppaF is degraded by trypsin while ppaF sequestered inside the microsomes remains shielded. When trypsin accessibility was examined in ERAD reactions at 23 and 37 °C using wild type microsomes, we found that the amount of ppaF decreased from 92 to 54% over a 40-min chase, in agreement with previous results and indicating export of 46% of the ppaF to the cytosol (5) (Fig. 3). On the contrary, the amount of trypsin-sensitive ppaF was low and remained constant in the kar2 microsomes; only 5–7% of ppaF was exported to the cytosol (Fig. 3). We concluded that ppaF remained sequestered inside the microsomes and that the mutations in KAR2 abrogate ERAD by preventing efficient ppaF export to the cytosol.

FIG. 1. \textit{KAR2} and \textit{CNE1} synthetically interact. Colonies derived from tetratype spores in crosses between the indicated \textit{kar2} mutants and the \textit{cne1} deleted strain were obtained, re-streaked, and then incubated at the indicated temperatures on YPD medium for 2–3 days. A, tetratype progeny from a cross between \textit{kar2–1} and \textit{cne1Δ}; B, tetratype progeny from a cross between \textit{kar2–113} and \textit{cne1Δ}. Growth curves from single colonies for each cross in A and B are shown in C and D, respectively. Saturated cultures were diluted into YPD medium and grown with shaking at 35 °C. \textit{Open circles, wild type; closed circles, cne1Δ; open triangles, kar2; closed triangles, kar2cne1Δ.}

the role of BiP in ERAD, we prepared ER-derived microsomes from an isogenic wild type strain and three temperature-sensitive \textit{kar2} strains, \textit{kar2–1}, \textit{kar2–113}, and \textit{kar2–133}. The \textit{kar2–113} mutation converts a phenylalanine at position 196 to a

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microsomes prepared from kar2–1 were translocated into kar2–1 and a wild type strain grown at the permissive temperature. Posttranslocation chase reactions were performed as described under “Experimental Procedures.” The products of the chase samples, collected at 0, 10, 20, and 40 min, were precipitated with trichloroacetic acid and resolved by SDS-18%/4 M urea polyacrylamide gel electrophoresis. Relative amounts of polypeptides were measured using the Bio-Rad Phosphor Analyses program. Reactions using wild type microsomes (circles) exhibited identical levels of ERAD at 23 and 37 °C. The degradation of A1PiZ is also proteasome mediated in mammalian cell lines (5, 50). The degradation of A1PiZ, a mutant form of the mammalian BiP, could be imported into the yeast ER but was subsequently degraded by the proteasome (5, 50).

To observe whether these mutations also affected the degradation of an ERAD substrate in vivo, the stability of A1PiZ was monitored by pulse-chase protein radiolabeling in the kar2–1, kar2–133, and wild type microsomes, and posttranslocation chase incubations were performed in the presence of wild type cytosol at either 23 °C (squares) or 37 °C (triangles). Samples were collected at 0, 10, 20, and 40 min, and the reactions were treated with trypsin and processed as described in the legend to Fig. 2. Reactions performed with wild type microsomes exhibited identical amounts of protected polypeptides. Reactions performed using wild type microsomes exhibited identical levels of ERAD at 23 and 37 °C (circles).

To determine whether the recovery of ERAD could be recapitulated in vitro, the degradation of A1PiF was monitored using microsomes prepared from wild type and kar2–1 cells cultured under growth conditions used for the in vivo experiments, conditions that led to elevated levels of Kar2–1p (Fig. 5). We found that 49% of A1PiF remained following a 10-min chase in kar2–1 microsomes, and 44% of A1PiF remained in the wild type microsomes. These results indicate that the ERAD defect was alleviated in both microsomes and cells cultured under conditions in which the level of Kar2–1p was induced.

Ssa1p Is Not Required for ERAD in Vitro or in Vivo—If soluble ERAD substrates are exported to the cytoplasm for degradation, there must be a driving force to expel these polypeptides. Because BiP has been proposed to drive polypeptides into the ER lumen by acting as a ratchet or a motor (reviewed in Ref. 33), one might imagine that a BiP homologue is required for polypeptide import into the ER (38, 39). Cytosolic hsp70s may also be required for ERAD by retaining a polypeptide in an unfolded conformation or by actively unfolding the polypeptide and ushering it into the proteasome: some polypeptides can be degraded in the absence of ubiquitination (1, 5, 15) and thus must be unfolded and/or targeted into the proteasome by an alternate mechanism. In addition, the degradation of polypeptides in bacteria requires specific chaperones that catalyze protein unfolding (53). By analogy, a chaperone activity in eukaryotes may be necessary for degradation because only unfolded proteins can interact with the
catalytic core of the proteasome (54), some of which may not be ubiquitinated (1, 5, 15).

To determine whether Ssa1p/2p was required for ERAD, we used a wild type SSA1 strain in which the homologous ssa2, ssa3, and ssa4 genes contained insertion mutations and an ssa1 temperature-sensitive strain (known as ssa1–45) in which the ssa2, ssa3, and ssa4 genes were similarly inactivated (38). Craig and co-workers (38) had previously shown that the translocation of pppF factor into the ER of ssa1–45 was inhibited after 2 min at 37 °C and found that the translocation block was complete. Thus, we grew both of these strains at a permissive temperature of 20 °C, and then one-half of each culture was shifted to 37 °C. As shown in Table I, we observed that cytosol prepared from the ssa1 temperature-sensitive strain was proficient for ERAD when present at a final concentration of 5 mg/ml, regardless of whether the culture used to prepare the cytosol had been grown at a nonpermissive temperature and regardless of the assay temperature. However, one explanation for this lack of an effect on ERAD is that saturating amounts of cytosol may have been used and that subtle defects in pppF degradation could have been obscured. For example, if Ssa1p acts with other components to facilitate ERAD, Ssa1p-dependent ERAD may be evident only if limiting amounts of cytosol are used. Therefore, we measured pppF degradation at varying concentrations of SSA1 and ssa1–45 cytosols from cells grown exclusively at the permissive temperature or that had been shifted to 37 °C for 45 min, and from an unrelated wild type yeast strain, RSY607. As shown in Fig. 5, we found that the degree of pppF degradation was similar at each concentration regardless of which cytosol preparation was used. These results suggest that the presence of wild type Ssa1p was not required for proficient ERAD in vitro.

By performing quantitative immunoblotting, we determined that Ssa1p comprises ~3% of the total protein in our cytosol preparations (data not shown). Because Ssa1p has been observed to associate with ppppF (55), it was also possible that excess Ssa1p may inhibit pppF degradation, perhaps by shielding the substrate from the proteasome. Thus, we performed an in vitro ERAD reaction using nonsaturating concentrations of cytosol (1.5 mg/ml) with the different cytosol preparations either in the absence or presence of a ~3-fold excess of Ssa1p (10% of total protein). However, as displayed in Table I, we discovered that ERAD was unaffected.

One concern was that the ssa1–45 cytosol used for these studies may not exhibit chaperone-dependent defects in vitro. To examine whether this was the case, we utilized an established assay in which the ability of cytosolic chaperones to refold heat-denatured firefly luciferase is measured (47). Because Ssa1p has been shown to directly facilitate the folding of firefly luciferase in vitro (56, 57), we chose to examine whether the ssa1–45 cytosols and the wild type cytosol could support heat-denatured luciferase refolding. In these studies, the denatured material must be “held” in a folding competent conformation until the cytosol is added (47). Because mammalian Hsp110 has previously been shown to fill this role, we used purified Sse1p, a yeast hsp110 homologue.2 As shown in Fig. 7, we discovered that the ssa1–45 cytosols were largely unable to refold luciferase, whereas the refolding activity in wild type cytosol was intact. Interestingly, the ssa1–45 cytosols were defective for this activity regardless of whether they had been prepared from cells shifted to 37 °C; similar results in which in vitro defects of temperature-sensitive mutants are more severe than those observed in vivo have been observed previously (37).

FIG. 4. Defects in Kar2p initially inhibit the in vivo degradation of A1PiZ. Pulse-chase protein radiolabeling experiments were performed in kar2–1, kar2–113, kar2–133 (closed symbols), and a wild type strain (open symbols) expressing A1PiZ as described under “Experimental Procedures.” Chase reactions were performed at either 23 °C (circles) or 37 °C (triangles and squares). A1PiZ immunoprecipitated from the chase reactions at 0, 30, and 90 min was resolved by 10% SDS-polyacrylamide gel electrophoresis, and the relative amounts of A1PiZ were determined using the Bio-Rad Phosphor Analyses program.

FIG. 5. Kar2p levels increase upon expression of A1PiZ at 37 °C. kar2–1 (triangles), kar2–113 (squares), kar2–133 (diamonds), and wild type cells (circles) expressing A1PiZ were incubated for the indicated times at either 23 °C (closed symbols) or 37 °C (open symbols) before cell lysates were prepared and immunoblots using anti-BiP antibody were performed. Relative amounts of BiP were obtained from scanned images using the Bio-Rad Phosphor Analyses program. Values represent the means of at least three independent experiments. Note that at 90 min, a precursor form of Kar2–113p was observed, consistent with the expected translocation defect in this strain (see text for details).
Specificity of Hsp70 Action in ERAD

Cytosols from a wild type and ssa1 temperature-sensitive mutant strain were prepared and added to a final concentration of either 1.5 or 5 mg/ml to microsomes containing pαF in the presence of an ATP regenerating system. The degradation of pαF was measured, as described under “Experimental Procedures” and in the legend to Fig. 2, after a 5-min reaction at the indicated temperatures. Values represent the mean percentages of pαF remaining of at least three independent experiments ± S.D.

![Graph showing ERAD dependence on cytosol concentration: ssa1 mutant cytosol is proficient for ERAD.](Image 7)

Nevertheless, these results indicate that the ssa1–45 mutant cytosols are defective for a known chaperone activity, and combined with the data presented above, they indicate that this activity is not required for ERAD.

To confirm that ERAD was Saa1p-independent, Saa1p-depleted cytosol was prepared from the MW141 strain. MW141 lacks the SSA1, SSA2, and SSA4 genes encoding the three major cytosolic hsp70s but carries the SSA1 gene on a galactose-regulated promoter (39, 40). Whereas growth of this strain for 8–10 h on 2% glucose leads to a 2-fold decrease in the amount of Saa1p and a strong defect in posttranslational protein translocation (39), growth of the strain on a 2% galactose/0.2% glucose mixture yields cytosol containing wild type levels of Saa1p as determined by quantitative immunoblotting.4 Thus, we prepared cytosol from MW141 grown under each condition to use in an in vitro ERAD assay. When we measured pαF degradation using the Saa1p-depleted cytosol or cytosol containing wild type levels of Saa1p at a range of concentrations, we found that ERAD activities were identical (data not shown). We also noted that the amount of pαF remaining at saturating cytosol concentrations was higher than in previous experiments (∼40%, as compared with ∼10%; see Fig. 6). This may reflect the altered cellular growth conditions used prior to the cytosol preparation or the absence of Saa2–4p (see under “Discussion”). These results further support our conclusion that Saa1p is not required for pαF degradation in vitro.

It was possible in these in vitro studies that wild type

![Graph showing ssa1–45 mutant cytosol is defective for refolding heat-denatured firefly luciferase.](Image 8)

Saa1p may have contaminated the microsomes used to assay the ssa1–45 cytosols, a supposition we confirmed by immunoblotting the microsomes with an Saa1p-specific antibody (Fig. 8). If Saa1p acts catalytically during ERAD, even low concentrations of membrane-bound wild type chaperone may have masked an ssa1 mutant effect on ERAD. Thus, we prepared microsomes from the ssa1 temperature-sensitive strain grown exclusively at the permissive temperature (filled circles), or ssa1–45 cells grown at the permissive temperature and then shifted to 37 °C for 45 min (open circles). Refolding reactions were conducted at 26 °C, and the final concentration of each cytosol was 4 mg/ml. Luciferase activity (defined by relative light units) was detected as described under “Experimental Procedures” at the indicated time points.

As shown above, mutations in KAR2 prevent export of pβF to
the cytosol for degradation; thus, we asked whether or not Ssa1p had a role in pOF export. A defect in export due to loss of Ssa1p activity may not have been observed in the experiments presented above if proteolysis is the rate-determining step in the in vitro ERAD assay. To this end, we monitored the pool of exported pOF using the trypsin protection assay and cytosol prepared from a wild type yeast strain and from ssa1–45 cells following incubation at either the permissive or the nonpermissive temperature. We found that the trypsin-sensitive pool of pOF in 15-min chase samples containing ssa1–45 cytosol was not significantly different than that seen with wild type cytosol (~75% of pOF protected in all cases).

Finally, to confirm further that ERAD was Ssa1p-independent and to determine whether Ssa1p was required for proficient ERAD in vivo, we measured the degradation of A1PiZ in the wild type and ssa1–45 strain at both the permissive temperature and during a 37 °C incubation. As shown in Fig. 9, we noted that the rate of A1PiZ degradation over a 90-min chase was similar in both strains when monitored at 23 or 37 °C.

DISCUSSION

The experiments reported here demonstrate that mutations in the gene encoding BiP inhibit the degradation of pOF in vitro and A1PiZ in vivo. We also discovered that defects in or depletion of the homologous cytoplasmic hsp70, Ssa1p, have no effect on ERAD, even though these conditions prevent protein import into the ER both in vivo (as determined by pulse-chase analyses) and in vitro (as shown using wheat germ-translated ppaF) (38, 39, 45). We show further that BiP is required to export pOF from the ER and that this is involved in the recognition of ERAD substrates and/or directly in the export process. In support of this first hypothesis, other reports indicated that mammalian BiP recognizes abnormally folded or unassembled polypeptides in the ER and targets them for degradation (24, 34, 35). A direct role for BiP in the export process is more difficult to imagine unless it regulates the translocation channel to permit access of ERAD substrates to the cytoplasm. In accordance with this view, the kar2–113 mutant that was used both by Plemper et al. (18) and in this report prevents an early event during protein translocation (36). Early events may include the transfer of the signal sequence-bearing nascent polypeptide to the translocation pore, Sec61p (reviewed in Ref. 33), or the gating of the pore. Defects in Sec61p gating are expected to compromise pOF or CPP*-degradation as the channel must support ERAD substrates to the cytosol. Indeed, Johnson and co-workers (58) have recently shown that BiP regulates the opening of the mammalian translocation channel during protein translocation into the ER. Whether yeast BiP similarly gates the translocation pore and whether Kar2–113p is unable to exhibit this activity are currently unknown.

We also show that mutations in KAR2 prevent the efficient degradation of A1PiZ in vivo and that the defect in A1PiZ degradation at the nonpermissive temperature can be rescued over time (Fig. 4). We suggest that this rescue may arise from the induction of BiP during the incubation (Fig. 5). We do not discount the alternative explanation that increased expression of other chaperones may rescue this defect, as incubating yeast at higher temperatures or expressing a misfolded secretory protein (such as A1PiZ) may increase the synthesis of some ER chaperones by the heat shock and unfolded protein stress responses, respectively. This response is not limited to yeast: hepatocytes from some individuals homozygous for A1PiZ and with chronic liver disease cannot degrade A1PiZ and induce the synthesis of BiP, the stress protein SP90, and ubiquitin (59, 60). In contrast, these proteins are not induced in heterozygous individuals or in those homozygous for A1PiZ but who exhibit normal liver function (59). This result indicates that when ERAD efficiently removes A1PiZ, no stress response is triggered.

We previously suggested that a cytosolic molecular chaperone may drive ERAD substrates from the ER by acting as a ratchet or molecular motor (reviewed in Ref. 1), and a likely candidate for this factor was Ssa1p because: 1) BiP performs this role during translocation into the ER (reviewed in Ref. 33) and BiP and Ssa1p are ~60% identical; and 2) Ssa1p is required to chaperone proteins into the ER (38, 39, 45, 55) and may unfold native proteins (52), and ERAD substrates may require this activity when delivered to the proteasome. In addition, cytosolic hsc70s in mammals are required for ERAD (see below). To our surprise, however, we discovered that ERAD is Ssa1p-independent. Thus, it is uncertain what protein drives substrates such as pOF and A1PiZ from the ER before degradation may ensue and whether other cytosolic chaperones are required to usher substrates to the proteasome. In this regard, we did note that ERAD was less efficient when measured in vivo in the SSA1 strain deleted for SSA2–4 (Fig. 9) than in a bona fide wild type strain (that contains SSA2–4) used in Fig. 4, and when cytosol that was depleted for Ssa1p, Ssa2p, and Ssa4p was used to support ERAD in vitro. These differences may arise from either the unique strain backgrounds used in the experiments or because Ssa2p and/or Ssa4p play supporting but nonessential roles during ERAD in vivo. Regardless, it is clear that the essential role of Ssa1p in protein translocation into the ER is not required for ERAD.

We suggest four scenarios by which ERAD substrates may be driven from the ER. First, unidentified cytosolic chaperones may associate with polypeptides as they emerge from the translocation channel and export them from the ER, acting either as a ratchet, or—if harnessed to the membrane—as a motor. Sec-

![Fig. 8. Ssa1p is associated with ER-derived microsomes. Cytosol and microsomes were immunoblotted for BiP and Ssa1p as described under “Experimental Procedures.” Whereas BiP was enriched about 20-fold in microsomes compared with cytosol, Ssa1p levels were similar in the two preparations. Lane 1, 1 μg of total protein; lane 2, 10 μg of total protein; lane 3, 25 μg of total protein.](image)

![Fig. 9. A temperature-sensitive defect in Ssa1p does not affect the in vivo degradation of A1PiZ. Pulse-chase protein radiolabeling experiments were performed in the ssa1 temperature-sensitive mutant (triangles) and an isogenic wild type strain (circles) at either 23 °C (closed symbols) or 37 °C (open symbols). A1PiZ immunoadsorbed from the chase samples at 0, 30, 60, and 90 min was resolved by 10% SDS-polyacrylamide gel electrophoresis. Relative amounts of A1PiZ were determined using the Bio-Rad Phosphor Analyses program. First order decay curves were generated with average values from at least three independent experiments to determine the half-life of A1PiZ.](image)
ond, ubiquitination may provide the favorable energy needed to dislocate ERAD substrates from the ER (10, 17, 61). Third, the BiP-See63p pair that is required for posttranslational translocation into the ER (reviewed in Ref. 33) may function in reverse to engineer the proteasome-mediated degradation of some ERAD substrates (10, 17, 61). Third, the BiP-Sec63p pair that is required for posttranslational translocation into the ER (reviewed in Ref. 33) may function in reverse to engineer the proteasome-mediated degradation of some ERAD substrates (10, 17, 61). Fourth, the BiP-Sec63p pair that is required for posttranslational translocation into the ER (reviewed in Ref. 33) may function in reverse to engineer the proteasome-mediated degradation of some ERAD substrates (10, 17, 61). Fourth, the BiP-Sec63p pair that is required for posttranslational translocation into the ER (reviewed in Ref. 33) may function in reverse to engineer the proteasome-mediated degradation of some ERAD substrates (10, 17, 61).

In contrast to the results presented here, a requirement for cytosolic hsp70s in the proteasome-mediated degradation of some mammalian proteins has been observed (48, 63). In one of these cases, the hsp70 cognate protein, hsc70, was required for the conjugation of ubiquitin onto protein substrates (63). Ssa1p may not be required in our system because we failed to note ubiquitination of pol α on route to its degradation (5). Therefore, it will be interesting to determine whether the proteolysis of CYP*, to which ubiquitin is attached before degradation (4, 10), requires Ssa1p.

Finally, the action of BiP and calnexin during ERAD remains to be fully elucidated. Both calnexin (30) and BiP (this report) may function in reverse to engineer the proteasome-mediated degradation of some ERAD substrates (10, 17, 61). Fourth, the BiP-Sec63p pair that is required for posttranslational translocation into the ER (reviewed in Ref. 33) may function in reverse to engineer the proteasome-mediated degradation of some ERAD substrates (10, 17, 61). Fourth, the BiP-Sec63p pair that is required for posttranslational translocation into the ER (reviewed in Ref. 33) may function in reverse to engineer the proteasome-mediated degradation of some ERAD substrates (10, 17, 61). Fourth, the BiP-Sec63p pair that is required for posttranslational translocation into the ER (reviewed in Ref. 33) may function in reverse to engineer the proteasome-mediated degradation of some ERAD substrates (10, 17, 61). 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Finally, the action of BiP and calnexin during ERAD remains to be fully elucidated. Both calnexin (30) and BiP (this report) may function in reverse to engineer the proteasome-mediated degradation of some ERAD substrates (10, 17, 61).