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Dependence of Endoplasmic Reticulum-associated Degradation on the Peptide Binding Domain and Concentration of BiP

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ER-associated degradation (ERAD) removes defective and mis-folded proteins from the eukaryotic secretory pathway, but mutations in the ER lumenal Hsp70, BiP/Kar2p, compromise ERAD efficiency in yeast. Because attenuation of ERAD activates the UPR, we screened for *kar2* mutants in which the unfolded protein response (UPR) was induced in order to better define how BiP facilitates ERAD. Among the *kar2* mutants isolated we identified the ERAD-specific *kar2-1* allele (Brodsky *et al.* J. Biol. Chem. *274,* 3453–3460). The *kar2-1* mutation resides in the peptide-binding domain of BiP and decreases BiP's affinity for a peptide substrate. Peptide-stimulated ATPase activity was also reduced, suggesting that the interdomain coupling in Kar2-1p is partially compromised. In contrast, Hsp40 cochaperone-activation of Kar2-1p's ATPase activity was unaffected. Consistent with UPR induction in *kar2-1* yeast, an ERAD substrate aggregated in microsomes prepared from this strain but not from wild-type yeast. Overexpression of wild-type BiP increased substrate solubility in microsomes obtained from the mutant, but the ERAD defect was exacerbated, suggesting that simply retaining ERAD substrates in a soluble, retro-translocation-competent conformation is insufficient to support polypeptide transit to the cytoplasm.

INTRODUCTION

Before being delivered to their ultimate locations, secreted proteins are monitored by a quality control "machine" associated with the endoplasmic reticulum (ER; reviewed by Ellgaard *et al.,* 1999); aberrant polypeptides may be retrotranslocated from the ER to the cytoplasm and destroyed by the proteasome in a process termed ER-associated degradation (ERAD; McCracken and Brodsky, 1996). The importance of defining the molecular mechanism of ERAD is underscored by the fact that several human diseases arise from the accumulation or accelerated degradation of ERAD substrates and because some bacterial toxins and viruses coopt the ERAD pathway to exert their effects (reviewed in Thomas *et al.,* 1995; Brodsky and McCracken, 1999; Aridor and Hannan, 2000; Fewell *et al.,* 2001).

ERAD may result from inefficient protein folding, so it is not surprising that molecular chaperones are required for this process. Chaperones prevent the formation of off-pathway intermediates or directly catalyze folding and have been proposed to "judge" whether a nascent protein will ultimately fold or whether it should be targeted for degradation (reviewed by Hayes and Dice, 1996; Hartl, 1996; Horwich *et al.,* 1999; Plemper and Wolf, 1999; Römisch, 1999; Wickner *et al.,* 1999; Fewell *et al.,* 2001; Höhfeld *et al.,* 2001). Hsp70 (heat shock proteins with a molecular mass of ~70 kDa) molecular chaperones hydrolyze ATP concomitant with the binding of peptides with overall hydrophobic character (Flynn *et al.,* 1991; Blond-Elguindi *et al.,* 1993; Rüdiger *et al.,* 1997); therefore, Hsp70s might retain the solubility of unfolded, retro-translocating polypeptides during their voyage from the ER to the cytoplasm via the Sec61p translocation channel or might "gate" this channel (Wiertz *et al.,* 1996;

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Pilon *et al.*, 1997; Plemper *et al.*, 1997; Johnson and Haigh, 2000; Tsai *et al.*, 2002).

In accordance with these hypotheses, several lines of evidence support a role for the lumenal Hsp70, BiP, in ERAD in both mammals and yeast. First, the proteolysis of ERAD substrates coincides with the rate at which they are released from BiP in the mammalian ER (Knittler *et al.*, 1995; Beggah *et al.*, 1996; Skowronek *et al.*, 1998; Chillaron and Haas, 2000), and BiP associates preferentially with exposed regions of an unfolded ERAD substrate (Schmitz *et al.*, 1995). Second, the degradation of an ERAD substrate is slowed when yeast contain a mutant allele in the gene encoding BiP, *KAR2* (*kar2-113*; Plemper *et al.*, 1997), and two ERAD substrates in yeast, CPY* and pαF, aggregate in microsomes prepared from another *kar2* mutant shifted to the nonpermissive temperature (*kar2–203*; Nishikawa *et al.*, 2001). The delivery of an ERAD substrate to the proteasome is also reduced in microsomes prepared from *kar2-1* and *kar2-133* mutants; however, in contrast to the *kar2* mutants used in these other studies, the *kar2-1* and *kar2-133* alleles compromise ERAD efficiency but do not affect protein translocation (import) into the ER, suggesting that the roles for BiP during protein translocation and retro-translocation are distinct (Brodsky *et al.*, 1999) and that a biochemical analysis of the corresponding Kar2-1 and Kar2-133 mutant proteins might elucidate which biochemical activities of BiP are required for ERAD.

To better define the action of BiP during ERAD we sought *kar2* mutants in which the unfolded protein response (UPR) was induced and identified the *kar2-1* allele. We also present a biochemical analysis of the Kar2-1 and Kar2-133 mutant proteins and the physiological consequences of Kar2-1/133p expression in yeast.

MATERIALS AND METHODS

Strains and Plasmids

Yeast strains used for these studies were as follows: MS10 (***a*** *ura3-52 leu2-3112 ade2-101*); MS1111 (***a*** *ura3-52 leu2-3112 ade2-101 kar2-1*); and MS193 (***a*** *ura3-52 leu2-3112 ade2-101 kar2-133*). The *kar2-1* mutant was first described by Polaina and Conde (1982) and the *kar2-133* mutant was obtained by plasmid shuffle, as outlined in Vogel *et al.* (1990). DNA sequence analysis identified the mutations in the *kar2-1* and *kar2-133* open reading frames. To obtain *kar2* mutants in which the UPR was activated, the following strains were constructed. First, a *KAR2* disruption cassette was obtained by amplifying the *HIS3* gene from pRS303 with primers BiPDel1 (5′-G G A C A C G A A A A G G G T T C T C T G G A A G A T A T A A A T A T G G C T A T G C T C T T G G C C T C C T C T A G-3′) and BiPDel2 (5′ C A C T G T T A C T G A G T A C C T T A A C C C C A G T C T C T A T A C T C T T C T C G T T C A G A A T G A C A C G-3′) and that contain a region of homology to the *KAR2* gene on each end. This disruption cassette was used to transform the yeast wild-type diploid strain YPH501 (**a/**α, *his3-*Δ*200/his3-*Δ*200, leu2-*Δ*1/leu2-*Δ*1, ura3-52/ura3-52, trp1-*Δ*63/trp1-*Δ*63*) and His+ transformants in which one *KAR2* allele was disrupted were confirmed by PCR using primers HISend (5′-GTGATTAACGTCCACACAGG-3′) and BiPend (5′-GGGATGAGATGAGATGAGATG-3′). The resulting strain was transformed with the single-copy, *KAR2*-containing plasmid, pMR397, which is marked with *URA3*. The transformed diploid strain was starved for nitrogen for 5 d to induce sporulation and tetrads were dissected and grown on rich medium. After replica plating the resulting colonies on various selective media to screen for auxotrophies, the MMY8–2 strain (α, *kar2::HIS3, his3-*Δ*200, leu2-*Δ*1, ura3-52, trp1-*Δ*63, pMR397*) was isolated. MMY8–2 was then transformed with pMZ11 (*CEN4, TRP1,* UPRE::LacZ) to monitor UPR activation through β-galactosidase activity (Zhou and Schekman, 1999). The resulting strain is called MMY9 and was used for the screen described below.

BiP was overproduced in wild-type and *kar2-1* yeast from the 2μ *URA*-marked plasmid, pMR109, which contained the *Cla*I-*Sal*I (*KAR2*) fragment from pMR48 (Rose *et al.*, 1989). Cells with pMR109 were selected on synthetic complete medium lacking uracil (SC-ura) and supplemented with glucose to a final concentration of 2%. All manipulations involving yeast were performed using standard protocols (Rose *et al.*, 1990).

Isolation of kar2 Mutants Exhibiting an Enhanced UPR

Plasmid pMR713 (*CEN4, LEU2, KAR2*) was mutagenized with hydroxylamine (Rose *et al.*, 1990) and transformed into MMY9 (see above). The resulting transformants, which grew on medium lacking tryptophan, uracil, and leucine, were replica-plated on 5-FOA at 30°C to select for yeast lacking pMR397. Approximately 35,000 transformants were screened for constitutive activation of the UPR by monitoring β-galactosidase activity at 30°C with an agarose overlay containing X-Gal (100 μg/ml) and 0.2% Sarkosyl as a permeating agent (Kabani *et al.*, 2000a). Candidate colonies were restruck to reconfirm UPR activation, and the corresponding pMR713 mutagenized plasmids from the rescreened colonies (~120) were isolated and individually transformed back into MMY9. After replica-plating on 5-FOA and testing for β-galactosidase activity to confirm that UPR activation was plasmid-based, 20 mutants were retained. These resulting pMR713 plasmids were also introduced into MMY8–2 and the transformants were used for further analysis (see below). As a control, MMY8–2 was transformed with untreated pMR713 and replica-plated on 5-FOA to cure yeast of the pMR397 plasmid and is referred to as wild type where indicated.

ERAD, Translocation, and UPR Assays

The degradation of unglycosylated proalpha factor (pαF) in yeast ER-derived microsomes was measured as described (McCracken and Brodsky, 1996). In brief, ^{35}S-labeled prepro-alpha factor (ppαF) lacking the core consensus glycosylation sites was translocated into microsomes, after which the microsomes were harvested, washed twice, and resuspended in a chase reaction either containing or lacking an ATP-regenerating mix and yeast cytosol at a final concentration of 5 mg/ml. Reactions were incubated at 30°C and were quenched after 20 min by the addition of trichloroacetic acid (TCA) to a final concentration of 20%. The percentage of pαF remaining was determined by phosphorimage analysis after SDS-PAGE of electrophoresed reaction products.

In vitro translocation assays were prepared as above except that after a 40-min translocation assay the reactions were divided and incubated in the presence or absence of trypsin in order to determine the amount of protected, and thus translocated, pαF. Samples were processed and translocation efficiency was calculated as described (Brodsky and Schekman, 1993). Translocation of preBiP and ppαF was assayed in vivo after incorporation of ^{35}S-methionine into total cellular protein and immunoprecipitation of BiP and ppαF using specific antiserum and Protein A-sepharose, as previously published (Morrow and Brodsky, 2001).

The UPR was assayed quantitatively by measuring β-galactosidase activity in extracts (Rose and Botstein, 1983) prepared from yeast transformed with pJC104 (kindly provided by Dr. Peter Walter, University of California, San Francisco). Plasmid pJC104 encodes the *lacZ* gene behind four repeats of the unfolded protein response element (UPRE; Mori *et al.*, 1992; Cox *et al.*, 1993).

Protein Purification and Other Biochemical Assays

Hexahistidine-tagged derivatives of wild-type, Kar2-1p, and Kar2-133p were generated and purified by nickel affinity and conven-

tional chromatography as described for other mutant forms of Kar2p (McClellan *et al.,* 1998). The proteins were estimated to be >95% pure as determined by SDS-PAGE and Coomassie Brilliant Blue staining. Single-turnover ATPase assays (Sullivan *et al.,* 2000) of [α-^{32}P]ATP-BiP complexes indicated that the rate of ATP hydrolysis by hexahistidine-tagged yeast BiP (0.12–0.21 min^{-1}) was similar to that reported for hexahistidine-tagged mammalian BiP (0.30 min^{-1}; Chevalier *et al.,* 1998). Steady state ATPase assays were performed as previously published (McClellan *et al.,* 1998).

The glutathione-*S*-transferase (GST)-Sec63p-J fusion protein was purified as described (Corsi and Schekman, 1997), and a GST-Jem1p-J was purified from the *Escherichia coli* TG1 strain transformed with pSNJ20 (S. Nishikawa S. and T. Endo, unpublished data). The pSNJ20 plasmid allows the expression of the C-terminal 115 residues of Jem1p fused at the C terminus of GST. The fusion protein also contains a hexahistidine-tag at the C terminus. Bacteria were grown in Luria broth (LB) containing 50 μg/ml ampicillin at 26°C to midlog phase and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the cells were incubated further for 1 h. The bacteria were harvested, washed with water, and resuspended in Buffer A (50 mM HEPES-KOH, pH 7.4, 5 mM β-mercaptoethanol, 200 mM NaCl, 10 mM imidazole). Lysozyme was added to a final concentration of 0.1 mg/ml and the cells were incubated on ice for 30 min. Protease inhibitors (phenylmethylsulfonylfluoride, 1 mM; leupeptin, 1 μg/ml; pepsatin A, 1 μg/ml) were added and the cells were disrupted six times by sonication for 30 s with a 1-min incubation on ice between each sonication. Triton X-100 was added to a final concentration of 0.1%, the broken cells were centrifuged for 10 min at 13,000 rpm in a Sorvall SA600 rotor, and the resulting supernatant was centrifuged at 20,000 rpm in a Sorvall SA600 rotor for 20 min. The cleared lysate was loaded onto a 5 ml Ni-NTA agarose column (Qiagen, Valencia, CA) that had been equilibrated in buffer A, and the column was washed with 30 ml of the following: (1) Buffer A; (2) Buffer A containing 1% Triton X-100 and 5% glycerol; (3) Buffer A containing 1 M NaCl and 5% glycerol; (4) Buffer A containing 5 mM $MgCl_2$, 5 mM ATP, and 300 mM NaCl; (5) Buffer A containing 0.5 M Tris-HCl, pH 7.4, and 300 mM NaCl; (6) Buffer A containing 300 mM NaCl, 5% glycerol, and 25 mM imidazole; and (7) Buffer A containing 300 mM NaCl, 5% glycerol, and 50 mM imidazole. The GST-Jem1p protein was eluted with a 15 × 15-ml linear gradient of Buffer A containing 300 mM NaCl, 5% glycerol, and imidazole from 50–250 mM; 1-ml fractions were collected and analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. Peak fractions were pooled and dialyzed against 20 mM HEPES-KOH, pH 7.4, 50 mM KCl, and 5% glycerol, and then snap-frozen in liquid nitrogen and stored at −80°C. The GST-Jem1p was >95% pure as assessed by Coomassie Brilliant Blue staining.

GST-fusion protein pull-down assays were performed as published (Kabani *et al.,* 2000b, 2002). Cell extracts for Western blots were prepared as reported (Morrow and Brodsky, 2001), and the proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (0.22-μM pore diameter; Schleicher and Schuell, Keene, NH). Quantitative immunoblots were performed using anti-BiP rabbit antiserum (Brodsky and Schekman, 1993) and anti-Sec61p rabbit antiserum (Stirling *et al.,* 1992), used as a loading control, both of which were decorated with ^{125}I-Protein A (Amersham Biosciences, Piscataway, NJ). Images were obtained and quantified using a Fuji phosphorimager and MacBas software (v. 2.4; Fuji Medical Systems, Stamford, CT). Carbonate extraction on ~1 equivalent of ER-derived microsomes was accomplished as described in Fujiki *et al.* (1982), and the pellet and supernatant fractions were analyzed by SDS-PAGE and immunoblot analysis using Enhanced Chemiluminescence (Pierce, Rockford, IL). The Sec63p-BiP complex was purified from octylglucoside-solubilized yeast microsomes by diethylaminoethane, Superose-6, and hydroxylapatite column chromatography as previously published (Brodsky and Schekman, 1993).

The oligomeric state of pαF was assessed as described (Nishikawa *et al.,* 2001). In brief, washed microsomes containing pαF were obtained after an in vitro translocation assay (see above), the membranes were solubilized with Triton X-100, and the clarified extract was loaded onto a 5–40% sucrose gradient in 0.1% Triton X-100 and centrifuged at 145,000 × *g* for 20 h at 4°C. After the gradient was fractionated pαF was detected and quantified by SDS-PAGE and phosphorimager analysis.

Spectroscopy

The CALLQSRLLLSAPRRAAATARY (APPY) peptide was synthesized, purified, labeled, and analyzed by mass spectrometry as described in Montgomery *et al.* (1999). The CLLLSAPRR (p5) peptide (Pierpaoli *et al.,* 1998) was synthesized by solid phase methods on PerSeptive Biosystems (Framingham, MA) automated peptide synthesizer and purified by reverse-phase chromatography on a C18 column and analyzed by mass spectrometry. The affinity of yeast BiP for F-APPY (fluorescein-labeled APPY) and the binding of AEDANS-labeled p5 to BiP were measured by fluorescence anisotropy as described by Montgomery *et al.* (1999) after incubating 0.02–0.05 μM of F-APPY or p5-AEDANS with the indicated concentrations of BiP overnight at 4°C. The BiP used for these experiments was dialyzed for 16 h against 20 mM HEPES, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 0.8 mM DTT and concentrated using Centricon microconcentrators (Amicon, Beverly, MA). Circular Dichroism (CD) spectroscopy of yeast BiP (final concentration 1.6 μM) was performed on an Aviv CD Spectrometer (Model 202; Lakewood, NJ) using a 0.1-cm path-length. The temperature of the cuvette was maintained at 26°C.

RESULTS

Isolation of UPR-activating kar2 *Mutants*

BiP is required for both ERAD and for protein translocation (import) into the ER (reviewed in Fewell *et al.,* 2001). Therefore, we desired allele-specific *kar2* mutants to more specifically define the action of BiP during ERAD. Our screen for such alleles is based on that used by Zhou and Schekman (1999) to isolate *sec61* mutants that were ERAD-defective but translocation-proficient, and the premise for the screen derives from the fact that defects in ERAD result in UPR induction (Cassagrande *et al.,* 2000; Friedlander *et al.,* 2000; Ng *et al.,* 2000; Travers *et al.,* 2000).

The MMY9 strain bears a chromosomal deletion of the *KAR2* gene and is viable because it contains pMR397, a single-copy uracil-selectable plasmid that expresses *KAR2* under the control of its own promoter (Figure 1). MMY9 also contains plasmid pMZ11 that can be used to report on the UPR by measurements of β-galactosidase activity (Cox *et al.,* 1993; Zhou and Schekman, 1999). A library of mutagenized *kar2* alleles (*kar2**) was then transformed into this strain and pMR397 was counterselected on medium containing 5-FOA. After β-galactosidase activity was determined using an agar-overlay (see MATERIALS AND METHODS), *kar2**-containing plasmids from candidate colonies were purified and their ability to induce the UPR was reconfirmed. Twenty of the retransformed mutants exhibited UPR activation and were retained for further analysis.

We next assayed the growth properties of the *kar2* mutants at various temperatures. Although each mutant exhibited wild-type growth at 30°C (Figure 2) and 35°C (our unpublished data), several strains were thermosensitive at 37°C (e.g., *kar2-51*), cryosensitive at 15°C (e.g., *kar2-40*), or both (e.g., *kar2-68*). Also shown in Figure 2 is the relative

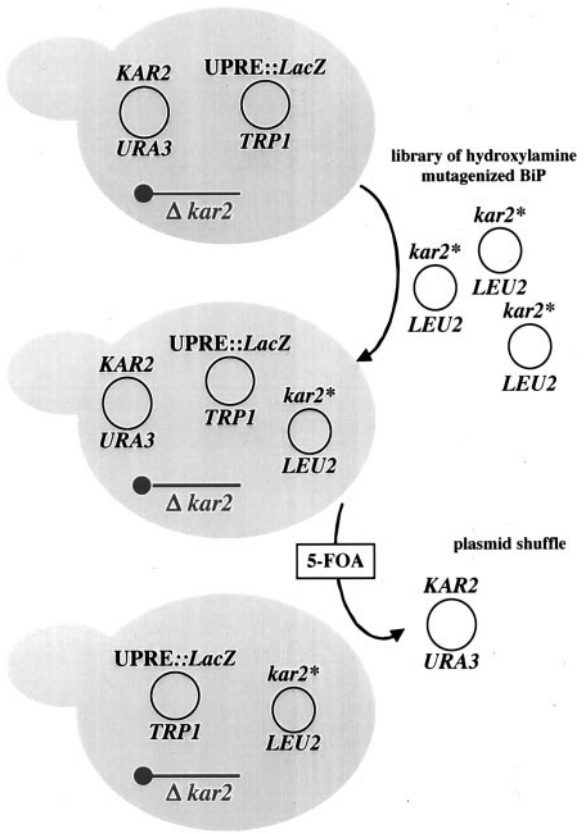

Figure 1. The screen for UPR-induced *kar2* mutant yeast (see text for details).

strain	15°C	30°C	37°C	UPR	mutation
KAR2				-	none
kar2-01				+++	ND
kar2-05				+++	P464F
kar2-09				+	A65T
kar2-22				++	P464R
kar2-24				+++	G171R,A510V,A533S
kar2-31				++	ND
kar2-37				++	ND
kar2-39				+++	ND
kar2-40				+	A194T
kar2-42				++	ND
kar2-47				+++	ND
kar2-49				+++	A415V
kar2-51				+++	P515L
kar2-57				++	ND
kar2-58				+	A203V,T629I
kar2-62				+	A203V
kar2-65				++	G42D
kar2-68				+	ND
kar2-78				+	G42D
kar2-79				++	ND

Figure 2. Temperature-sensitive phenotypic analysis of the UPR-inducing *kar2* mutants. Serial dilutions from log-phase cultures of the wild-type and the indicated mutant strains were inoculated on rich medium and incubated at the indicated temperatures for 3–6 d. The relative strength of UPR activation in the various mutants at 30°C is also indicated; −, no activation; + to +++, varying degrees of UPR activation as determined by the length of time required for the mutant colonies to turn blue in agar-overlay plate assays for β-galactosidase activity (see MATERIALS AND METHODS for details). The nomenclature of the mutants in this figure does not correspond to previously isolated *kar2* mutant alleles, but the amino acid position(s) and predicted substitution(s) in the mutant alleles are indicated. Nucleotide substitutions that do not change the encoded amino acid sequence from the wild-type allele (i.e., silent mutations) are not shown. ND, DNA sequence analysis of the mutant allele was not completed.

strength of the UPR activation in each strain, although no obvious correlation between growth phenotype and UPR was observed. For example, both *kar2-49* and *kar2-51* exhibit high UPR activation, whereas only *kar2-51* is thermosensitive (Figure 2).

To assay protein translocation, we examined whether cytoplasmic precursors of two secreted proteins, prepro-α-Factor (ppαF) and preBiP, accumulated in each strain at 26°C or after a 5-min shift at 37°C. ppαF is translocated posttranslationally into the ER, whereas BiP uses both the co- and posttranslational pathways (Ng *et al.*, 1996). As shown in Figure 3 the *kar2* mutants displayed varying translocation defects, ranging from no defect at 26°C and a very mild defect after temperature shift (e.g., *kar2–51*) to a strong block at 26 and 37°C (e.g., *kar2-40*). Interestingly, a band corresponding only to preBiP was observed in the *kar2-65* and *kar2-78* mutants; a similar result was obtained when the molecular mass of BiP was examined in ER-derived microsomes prepared from these strains by immunoblot analysis (our unpublished data). This result suggested that the Kar2-78 and Kar2-65 mutant proteins might possess an uncleaved signal sequence. Indeed, DNA sequence analysis revealed that both *kar2-65* and *kar2-78* bear a mutation of glycine 42 to an aspartic acid at the predicted signal sequence cleavage site (Rose *et al.*, 1989; Figure 2).

To determine whether retention of a signal sequence affected BiP solubility, we performed sodium carbonate extraction on microsomes obtained from the wild-type and *kar2-01* strains (in which signal sequence cleavage was apparent; Figure 3) and the *kar2-65* and *kar2-78* strains. Wild-type BiP and Kar2-01p were exclusively or predominantly found in the soluble fraction, as expected; in contrast, Kar2-65p and Kar2-78p were primarily membrane-associated. Moreover, we noted that ~50% of Kar2-65p and Kar2-78p were sensitive to exogenous protease when microsomes were prepared from strains expressing these mutant alleles (our unpublished data). Because a strong ppαF translocation defect was noted in *kar2-65* and *kar2-78* yeast (Figure 3), we suggest that a proportion of the signal sequence-containing BiP mutant proteins are incompletely translocated or fail to translocate and/or that BiP activity is reduced when its signal sequence is retained. In either case, both translocation and retro-translocation (ERAD) would be compromised and the UPR would be activated.

The kar2-51 *Mutant Bears the Same Amino Acid Substitution as the ERAD-specific* kar2-1 *Mutant*

On the basis of the results presented in Figures 2 and 3, we identified several mutants that were translocation-defective and might have exhibited an induced UPR because of fur-

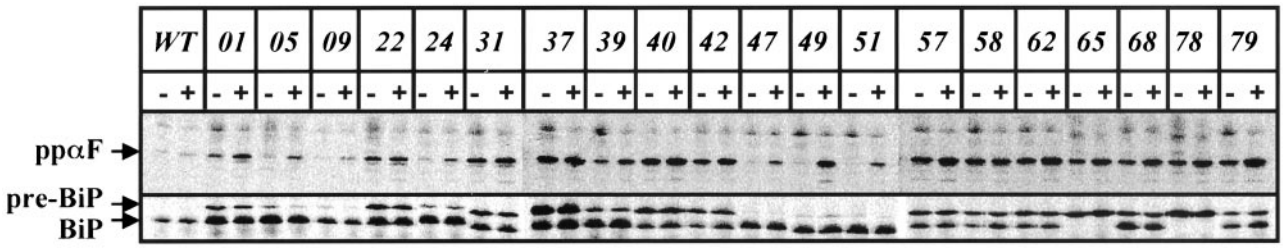

Figure 3. Analysis of ppαF and preBiP translocation in the *kar2* mutant strains. Each strain was grown at 26°C to midlog phase and total protein was labeled with ^{35}S-methionine/cysteine for 5 min at either 26° (−) or 37°C (+). Cell extracts were prepared and ppαF and BiP were immunoprecipitated, resolved by SDS-PAGE, and visualized by phosphorimager analysis.

ther effects on ERAD and/or protein folding. For example, defects in ERAD, protein folding, and translocation were previously noted for *kar2* mutant strains containing amino acid substitutions in the ATPase domain (e.g., *kar2-113* and *kar2-159*; Brodsky *et al.*, 1995; Simons *et al.*, 1995; Plemper *et al.*, 1997; see below). Consistent with this result, DNA sequence analysis uncovered an A203V mutation in *kar2-62* and an A194T mutation in *kar2-40* (Figure 2), both of which affect amino acids in BiP's ATPase domain.

We also identified a few strains that were translocation-proficient at permissive temperatures but exhibited an increased UPR, two criteria expected for ERAD-specific *kar2* mutants. Several of these candidates possess >1 amino acid changes as determined by DNA sequence analysis of the inserts in the corresponding *kar2*-containing plasmids (our unpublished data). However, we identified a point mutation of proline 515 to a leucine in *kar2-51*, which is the same mutation found on the chromosome in *kar2-1* yeast, a previously identified ERAD-specific mutant (Brodsky *et al.*, 1999). To confirm that yeast containing the plasmid-borne *kar2-51* mutation were also ERAD-defective but proficient for translocation, we performed in vitro translocation and ERAD assays with microsomes from the wild-type and *kar2-51* strains. As a control, we assayed these processes in microsomes prepared from the *kar2-159* strain, which contains a mutation in the ATPase domain, severely affecting the ability of Kar2-159p to bind ATP, and which consequently is defective for both ERAD and translocation (Brodsky *et al.*, 1995, and our unpublished data; see MATERIALS AND METHODS). We found that the ppαF translocation efficiency in microsomes derived from wild-type and *kar2-51* yeast were identical (~30%; Figure 4A), whereas the translocation efficiency in *kar2-159*–derived microsomes was ~9%. Microsomes obtained from either *kar2-51* or *kar2-159* yeast exhibited reduced retro-translocation and degradation of unglycosylated pαF, an in vitro ERAD-substrate (Figure 4B; McCracken and Brodsky, 1996). We conclude that *kar2-51* is ERAD-defective but translocation-proficient, thus supporting the premise of our screen.

For the remainder of this study, we chose to focus on the *kar2-1/kar2-51* mutant and on the *kar2-133* mutant, which is also ERAD-defective but translocation-proficient (Brodsky *et al.*, 1999). A further analysis of the other mutants will be reported elsewhere.

The kar2-1 *and* kar2-133 *Mutations Reside in the Peptide-binding Domain*

DNA sequence analysis of the *kar2-1* and *kar2-133* mutations indicates that a proline at position 515 (P-470 in DnaK) is converted to a leucine and a threonine at position 473 (T-428 in DnaK) is replaced by a phenylalanine, respectively. These residues are highly conserved among Hsp70s and lie near the tips of loops that connect β-sheets and that in turn forge the platform of the Hsp70 substrate-binding domain (Figure 5; Zhu *et al.*, 1996; our unpublished data). The mutation in *kar2-1* is in loop 6, whereas in *kar2-133* the mutation is in loop 3. Notably, the side chain of the threonine mutated in *kar2-133* is predicted to form a hydrogen bond to the polypeptide backbone at the tip of loop 5,6. Thus, we propose that the *kar2-1* and *kar2-133* mutations affect the structure of the peptide-binding domain similarly.

Yeast Expressing Kar2-1p and Kar2-133p Induced the Unfolded Protein Response

The strain used for the screen in which *kar2-1* was reisolated contained *kar2* on a single-copy plasmid. To ensure that experiments were free of potential plasmid copy-number artifacts, subsequent in vivo experiments were conducted with haploid yeast harboring a chromosomal copy of *kar2-1*.

Table 1. Kar2-1p and Kar2-122p expression induces the UPR

Strain/protein	*KAR2*/ BiP	*kar2-1*/ Kar2-1p	*kar2-133*/ Kar2-133p
UPR[a] (26°C)	840	6010	4910
(37°C)	720	7220	6280
Relative amount[b] (26°C)	1.0	2.0	3.0
(37°C)	1.0	2.3	4.5

[a] The UPR (in calculated units) in the indicated strains was measured from cells that were grown exclusively at 26°C or that had been shifted to 37°C for 1 hr, as described in the "MATERIALS AND METHODS".

[b] The amount of BiP, Kar2-1p, and Kar2-133p was measured in cell extracts by quantitative immunoblot analysis from strains that were grown exclusively at 26°C or that had been shifted to 37°C for 1 hr.

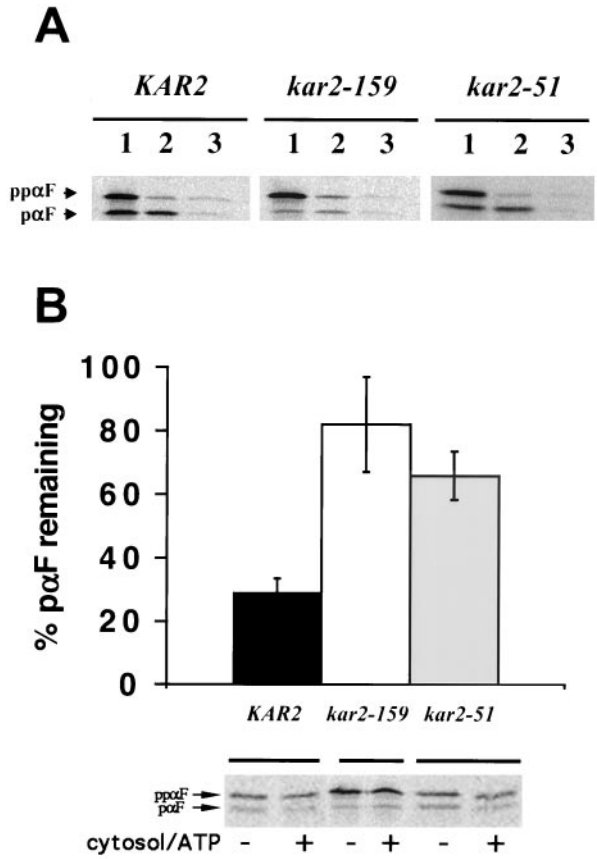

Figure 4. The *kar2-51* mutant (*kar2-1*) is translocation-proficient but ERAD-defective in vitro. (A) Microsomes derived from the indicated strains were incubated with in vitro–translated ^{35}S-labeled ΔGppαF (McCracken and Brodsky, 1996) for 40 min at 30°C. Equal amounts of each reaction were either (1) untreated or treated with (2) trypsin or (3) trypsin and Triton X-100, and incubated on ice for 30 min before proteins were TCA-precipitated and resolved by SDS-PAGE and visualized by phosphorimager analysis. The amount of trypsin-protected (translocated) signal sequence-cleaved pαF was 30, 9 and 28% in wild-type, *kar2-159*, and *kar2-51* microsomes, respectively (as averaged from three independent experiments). A representative experiment is shown. (B) ^{35}S-labeled ΔGppαF was translocated into microsomes derived from the indicated strains for 1 h at 20°C, the microsomes were washed, and cytosol and ATP were added to one half of the reactions, whereas the other half was incubated with buffer. After 20 min at 30°C the reactions were quenched with TCA, and precipitated proteins were resolved by SDS-PAGE and phosphorimager analysis to determine the percentage of pαF degraded. The buffer control was set to 100% in each experiment. Data represent the means of three independent experiments (±SD). A representative gel from one experiment is also shown.

The *kar2-133* strain and wild-type yeast were examined in parallel. UPR induction in the *kar2-1* and *kar2-133* mutants was confirmed after introduction of a UPR reporter plasmid (see MATERIALS AND METHODS): The UPR was enhanced 6–7-fold in the mutant strains compared with wild-type yeast when cells were grown at 26°C and was activated further after a shift to 37°C for 1 h (Table 1). In accordance with these data, we observed that the levels of Kar2-1p and

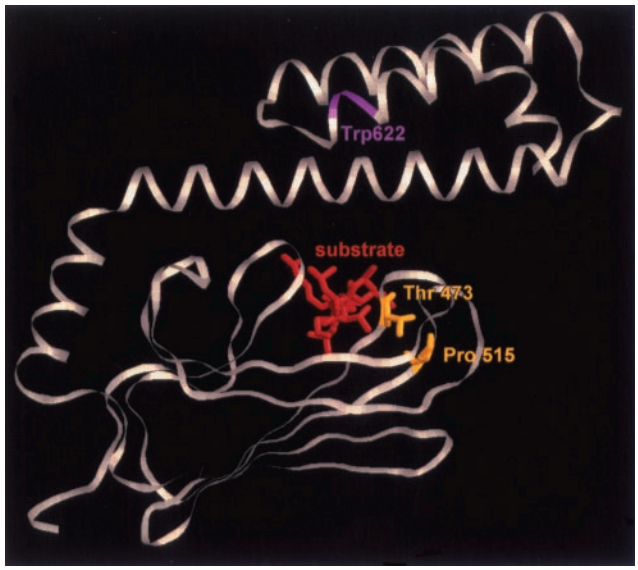

Figure 5. Mutations in *kar2-1* and *kar2-133* reside in the peptide-binding domain of BiP. The yeast BiP and *E. coli* DnaK amino acid sequences were aligned and mutated residues in *kar2-1* (P515L) and *kar2-133* (T473F) were mapped onto the three-dimensional structure of the DnaK peptide-binding domain (Zhu *et al.*, 1996). The mutated residues are highlighted in yellow, and the approximate location of the lone tryptophan (at position 622) in yeast BiP is indicated in magenta. The bound substrate is displayed in red and is oriented perpendicular to the page.

Kar2-133p were ≥2-fold higher than wild-type BiP in microsomes prepared from the respective strains and that this difference became more pronounced after a 37°C shift.

The Purified Kar2-1 and Kar2-133 Proteins Are Not Grossly Mis-folded

To begin to elucidate the molecular basis of the *kar2-1* and *kar2-133* ERAD defects we purified wild-type BiP, Kar2-1p, and Kar2-133p by nickel affinity and ion exchange column chromatography (see MATERIALS AND METHODS), and then we performed two assays to assess the global conformation of the mutant proteins. First, CD spectroscopy was performed and the spectral characteristics for the wild-type and mutant proteins were identical (our unpublished data). Second, we examined the digestion patterns of wild-type BiP and Kar2-1p and Kar2-133p after limited proteolysis in the presence of ATP or ADP and observed that the mutant proteins, like wild-type BiP, exhibited an ATP-dependent conformational change (McClellan *et al.*, 1998; J. Endres and J.L.B., unpublished data); these results are consistent with the fact that the single turnover and steady-state ATPase activities of wild-type BiP and Kar2-1p and Kar2-133p are similar (see MATERIALS AND METHODS; Figures 6 and 7; below).

Kar2-1p and Kar2-133p Interact Functionally with the J Domains of Sec63p and Jem1p

The interaction between BiP and the J-domain of Sec63p in the ER is essential for protein translocation (Brodsky and

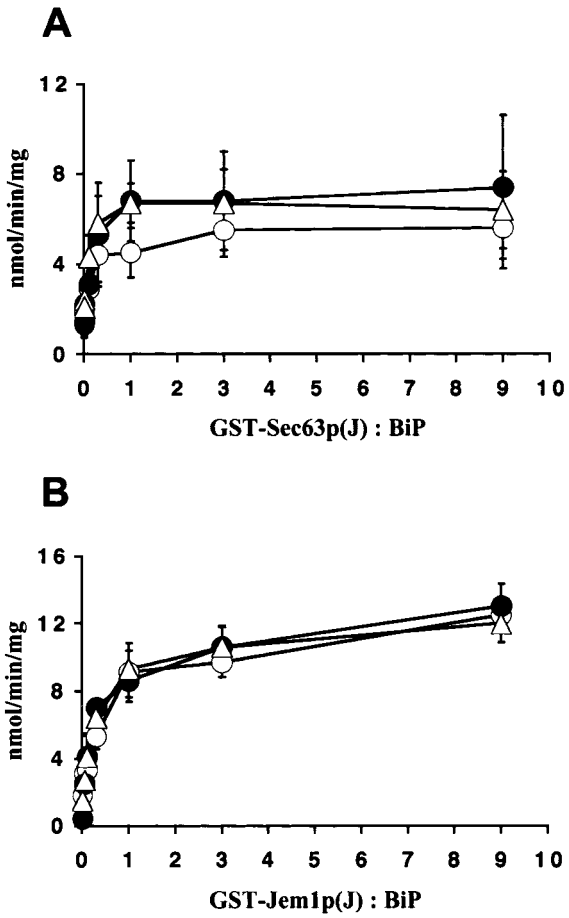

Figure 6. Kar2-1p and Kar2-133p interact functionally with the J domains of Sec63p and Jem1p. Steady state hydrolysis of ATP by wild-type BiP (○), Kar2-1p (●), and Kar2-133p (△) was measured using 5 μg of BiP and either the (A) GST-Sec63p-J or (B) GST-Jem1p-J fusion proteins. ATPase activity is plotted as nmol of ATP hydrolyzed/min/mg BiP vs. the molar ratio of the J domain–containing fusion proteins to BiP.

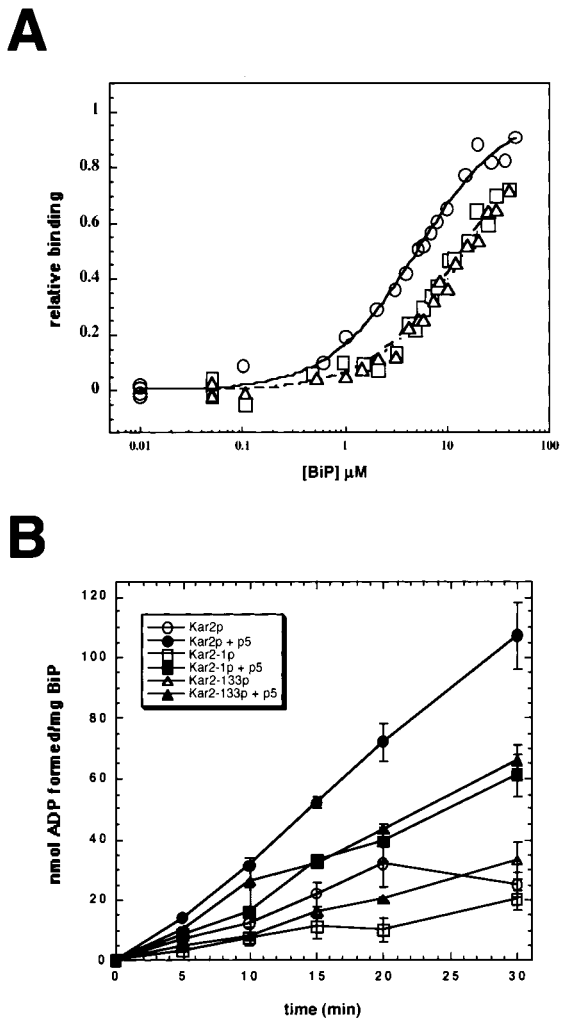

Figure 7. Kar2-1p and Kar2-133p exhibit reduced peptide affinity and interdomain coupling. (A) Fluorescence anisotropy measurements of F-APPY binding to wild-type BiP (○), Kar2-1p (□), and Kar2-133p (△), and a best-fit analysis of the data using a single binding site was performed as described by Montgomery *et al.* (1999). (B) Steady state ATPase assays were performed after assembling reactions on ice in either the presence (filled symbols) or absence (open symbols) of a 1000-fold molar excess of p5: Wild-type BiP (circles), Kar2-1p (squares), and Kar2-133p (triangles).

Schekman, 1993; Brodsky *et al.*, 1995; Lyman and Schekman, 1995; Corsi and Schekman, 1997; Matlack *et al.*, 1997; McClellan *et al.*, 1998), and a mutation in *SEC63* that abrogates this interaction has been reported to modestly reduce the degradation of a soluble ERAD substrate (Plemper *et al.*, 1997). Therefore, to determine if Kar2-1p and Kar2-133p associate functionally with the J domain of Sec63p, the wild-type and mutant proteins were incubated with increasing concentrations of a GST-Sec63p-J domain fusion protein that was used previously to report on the interaction of BiP with Sec63p (Corsi and Schekman, 1997; McClellan *et al.*, 1998). We found that the steady state ATPase activities of wild-type BiP and Kar2-1p and Kar2-133p were enhanced identically in a concentration-dependent manner by the Sec63p J domain (Figure 6A). We also assessed the ability of wild-type, Kar2-1p and Kar2-133p to associate with Sec63p after purification of the BiP-Sec63p-Sec71p-Sec72p complex from detergent-solubilized microsomes prepared from wild-type, *kar2-1*, and *kar2-133* mutant yeast (Brodsky and Schekman, 1993). After ion exchange (DEAE) and gel filtration (Superose-6) column chromatography, the complex eluted from hydroxylapatite on a 0.2–0.5 M phosphate gradient and the ratio of BiP to Sec63p that coeluted was comparable regardless of whether the microsomes derived from the wild-type or *kar2* mutant yeast (our unpublished data).

BiP also associates with two other J domain–containing proteins in the ER, Scj1p and Jem1p. Deletion of both proteins, but neither one alone, attenuates ERAD and increases ERAD substrate aggregation, suggesting that Scj1p and Jem1p function redundantly to facilitate polypeptide retro-

translocation (Nishikawa *et al.*, 2001). To measure the interaction of Jem1p with wild-type and the mutant Kar2 proteins, we incubated increasing concentrations of a GST-Jem1p-J domain fusion protein and measured steady state ATP hydrolysis, as above. The results shown in Figure 6B indicate that the J domain of Jem1p also activated the ATPase activities of each protein identically.

Kar2-1p and Kar2-133p Exhibit a Reduced Peptide Affinity and Compromised Peptide-stimulated ATPase Activity

BiP binds polypeptides in the lumen of the yeast ER to drive protein translocation (Sanders *et al.*, 1992; Matlack *et al.*, 1999), to engineer protein folding (Te Heesen and Aebi, 1994; Simons *et al.*, 1995), and to facilitate ERAD (Gillece *et al.*, 1999; Nishikawa *et al.*, 2001). We therefore wished to define whether the affinities of the wild-type and mutant proteins for a peptide substrate differed. Using a fluorescein-labeled peptide (F-APPY), increasing concentrations of the proteins, and measurements of fluorescence anisotropy to detect F-APPY-BiP complexes (Montgomery *et al.*, 1999), we found that the peptide affinities for wild-type BiP (5.1 ± 0.21 μM), Kar2-1p (13.9 ± 0.60 μM), and Kar2-133p (15.4 ± 0.41 μM) differed by 2.7–3.0-fold (Figure 7A). Because saturation was not achieved for the mutant proteins (yeast BiP aggregates in vitro at concentrations required to achieve saturation; our unpublished data), the calculated K_Ds for Kar2-1p and Kar2-133p are likely an under-estimate of the true K_Ds. Therefore, the peptide affinities for the ERAD-defective Kar2 mutants are significantly reduced relative to wild-type BiP. Furthermore, the peptide affinity for wild-type BiP is similar to that reported for the cytoplasmic yeast Hsp70, Ssa1p (~5 μM; Pfund *et al.*, 2001), which is 63% identical to Kar2p, supporting the efficacy of using this experimental method.

Hsp70-peptide interactions are controlled by interdomain coupling between the ATPase and peptide-binding domains (see for example, Ha *et al.*, 1997; Davis *et al.*, 1999). If the coupling between these domains is compromised, then the regulation of peptide binding and/or release may be altered and the delivery of an ERAD substrate to the cytoplasm would be attenuated. To assess interdomain coupling of wild-type BiP and the Kar2-1 and Kar2-133 proteins, we first needed to identify a more soluble, but related peptide substrate for yeast BiP. The peptide ultimately chosen, known as p5, is a shorter derivative of APPY (Pierpaoli *et al.*, 1998) and when labeled with the fluorescent reporter, AEDANS bound to the wild-type and mutant proteins, as measured by fluorescence anisotropy (our unpublished data). Next, we added saturating concentrations of unlabeled p5 (700 μM peptide to 0.7 μM BiP) and measured steady state ATP hydrolysis overtime. Although the endogenous ATPase activities of wild-type and the mutant Kar2 proteins were similar (Figures 6 and 7B), we observed an ~twofold reduced p5-mediated activation of ATP hydrolysis for the mutant proteins compared with wild-type BiP (Figure 7B). This second result suggests that the interdomain coupling between peptide interaction and ATP hydrolysis is altered to some degree in the Kar2 mutants.

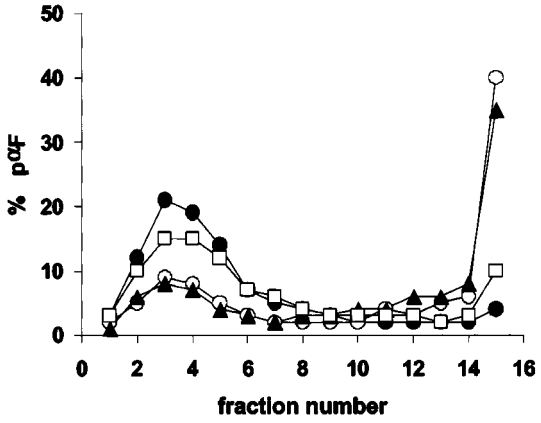

Figure 8. PαF aggregation is enhanced in *kar2-1*–derived mutant microsomes but can be resolubilized by wild-type BiP. ΔGppαF was translocated into microsomes derived from wild-type (●), *kar2-1* (○), and *kar2-133* (▲) cells, and *kar2-1* yeast transformed with a *KAR2* overexpression plasmid (pMR109; □). The microsomes were washed and incubated at 37°C for 30 min before Triton X-100 extraction and sedimentation in a 5–40% sucrose gradient. The percentages of pαF, the in vitro ERAD substrate, in fractions from the top (fraction 1) to the bottom (fraction 14) of the gradient are shown.

Wild-type BiP Increases the Solubility of an ERAD Substrate in **kar2-1** *Microsomes But Exacerbates the ERAD Defect*

BiP retains ERAD substrates in an extended conformation as they retro-translocate from the ER, and defects in BiP function can lead to substrate aggregation (Nishikawa *et al.*, 2001). To examine whether the solubility of an ERAD substrate was reduced in the ER of the *kar2* mutants, we measured the oligomeric state of pαF. Microsomes containing radiolabeled pαF were treated with detergent, the extract was centrifuged on a continuous sucrose gradient, and the amount of pαF in each fraction was measured after SDS-PAGE and phosphorimager analysis (Nishikawa *et al.*, 2001). When the sedimentation of pαF derived from wild-type microsomes was examined, we found that ~5% of the total pαF resided at the bottom of the gradient, whereas ~40% of the pαF resided at the bottom of the gradients when *kar2-1*– and *kar2-133*–derived microsomes were used, respectively (Figure 8). These data suggest that the propensity of pαF to aggregate in the ER of *kar2-1* yeast is enhanced, an effect that would hinder retro-translocation. We then examined pαF solubility in extracts prepared from *kar2-1* mutant yeast that had been transformed with a wild-type *KAR2* expression vector (pMR109) and observed that pαF was now largely absent from the bottom of the gradient and that the percentage of soluble pαF (fractions 2–5) increased.

To explore whether increasing pαF solubility enhanced its degradation, we performed the in vitro ERAD assay using microsomes from the wild-type and *kar2-1* strains containing either pMR109 or a vector control. The concentration of BiP was greater in microsomes prepared from the wild-type and *kar2-1* strains containing pMR109 compared with yeast harboring the vector control: BiP levels were elevated by ~50% when pMR109 was present in the wild-type strain and by

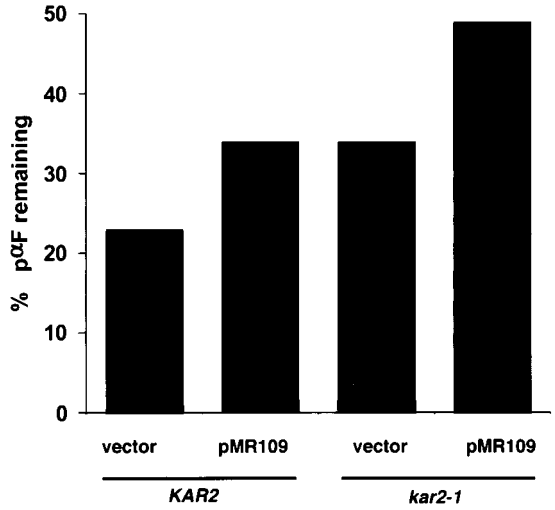

Figure 9. Increasing the concentration of wild-type BiP exacerbates the *kar2-1* ERAD defect and reduces ERAD efficiency in wild-type microsomes. Wild-type (*KAR2*) and *kar2-1* mutant microsomes prepared from strains harboring a control vector (vector) or overexpressing BiP (pMR109) were prepared and an in vitro ERAD assay was performed as described in MATERIALS AND METHODS. Data represent the means of three independent experiments and standard deviations are <10% of the means.

~90% when the same plasmid was present in the *kar2-1* strain as assessed by quantitative immunoblot analysis (our unpublished data). We then observed that microsomes from the *kar2-1* strain degraded pαF less efficiently than microsomes from the isogenic wild-type strain, as shown previously (Brodsky *et al.*, 1999; also see Figure 4B). Surprisingly, ERAD was inhibited by 40–50% in microsomes prepared from either strain containing the wild-type BiP expression vector compared with the vector control (Figure 9). This result suggests that the *kar2-1* mutation may be dominant with respect to ERAD because the ERAD defect was not rescued by expression of wild-type protein; however, it is formally possible that wild-type BiP failed to rescue the ERAD defect in *kar2-1*–derived microsomes because the amount of BiP may be too high in this system. Regardless, these data show that increasing the amount of BiP augments polypeptide solubility in a *kar2* mutant but is insufficient to restore ERAD to wild-type levels. Furthermore, increasing the amount of BiP in microsomes prepared from wild-type cells inhibited ERAD. A model for these observations is presented below.

DISCUSSION

To better define the role of BiP during ERAD, we devised a genetic screen to obtain ERAD-specific *kar2* mutants and isolated the *kar2-1* allele, which was previously coopted to show that BiP is required for the degradation of soluble (ER lumenal) ERAD substrates but not for the proteolysis of integral ER membrane proteins (Brodsky *et al.*, 1999). The position of the mutated residue in Kar2-1p suggested that another previously isolated *kar2* mutant, known as *kar2-133*, would exhibit similar properties. Indeed, *kar2-1* and *kar2-133* yeast are defective for the ERAD of three soluble substrates (CPY*: Zhang *et al.*, 2001; pαF and AiPiZ: Brodsky *et al.*, 1999), and we report here that expression of Kar2-1p or Kar2-133p induces the UPR and leads to the aggregation of one of these substrates. Furthermore, the Kar2-1 and Kar2-133 proteins exhibit reduced peptide affinities compared with wild-type BiP, and the addition of peptide fails to enhance ATP hydrolysis by the mutant proteins to the same degree as for wild-type BiP. This effect, although partial, most likely results from interdomain communication defects in the mutant proteins because an equal degree of J domain–stimulated ATPase activity was observed for Kar2-1/133p and wild-type BiP. Although we cannot formally exclude the possibility that the reduced peptide affinity of the mutants was the cause of lower peptide-stimulated ATPase activity, we note that the concentration of peptide used in this latter experiment was 1000-fold greater than the concentration of BiP, and—if the affinities of BiP for APPY and p5 are comparable—was >40-fold higher than the predicted K_D.

In light of these data, why are Kar2-1p– and Kar2-133p–expressing cells and microsomes derived from these strains translocation-proficient? The ability of BiP to bind a translocating polypeptide is essential to drive protein import, whether through its action as a "motor" or as a "ratchet" (Sanders *et al.*, 1992; Matlack *et al.*, 1999). However, it was previously noted by Rapoport and colleagues that 6–7 BiPs bind ppαF, and a BiP mutant lacking the peptide binding domain "lid"—and that exhibits a faster off-rate for peptide when ADP-bound (Misselwitz *et al.*, 1998)—supported ratcheting, but higher concentrations of the protein were required (Matlack *et al.*, 1999). Thus, a BiP mutant with a lower peptide affinity should catalyze protein translocation as long as greater amounts of the protein are present. In fact, this may be the case in *kar2-1* and *kar2-133* yeast and in Kar2-1/133p-containing microsomes (Table 1). We further note that the positions of the *kar2-1* and *kar2-133* mutations are likely to weaken interactions between the lid and the loops in the peptide-binding domain, resulting in proteins with similar properties as the "lid-less" BiP mutants used by Matlack *et al.* (1999); as expected, Hsc70 mutants with predicted weakened interactions between the loops and the lid exhibit an increase in peptide off-rates (Hu *et al.*, 2002; see below). In contrast, a BiP mutant in which peptide binding is completely abolished should be lethal and would not be isolated in our screen: Mutations in DnaK that significantly reduce the chaperone's peptide affinity cannot complement the Δ*dnaK* phenotype (for example, see Burkholder *et al.*, 1996). Therefore, we propose that *kar2-1* and *kar2-133* yeast are viable and translocation-proficient because they can balance the reduced affinity between BiP and peptides by synthesizing greater amounts of BiP via the UPR.

To explain the fact that *kar2-1* and *kar2-133* cells and microsomes obtained from these strains are ERAD-defective, we note that pαF aggregates in *kar2-1*– and *kar2-133*–derived microsomes (Figure 8), a phenomenon that would preclude retro-translocation through the pore formed by the Sec61p complex in the ER membrane. We suggest further that nucleotide and/or cochaperone-mediated pαF release from Kar2-1/133p is altered. For example, if pαF is released prematurely, then the polypeptide may aggregate before it can be retro-translocated. Several lines of evidence support

this view. First, expression of wild-type BiP restores pαF solubility (Figure 8), suggesting that it binds Kar2-1/133p–released polypeptides. Second, mutations in bovine Hsc70 in which interdomain coupling is altered exhibit an increase in peptide off-rate (Ha *et al.,* 1997). Third, conversion of glycine-468 to aspartic acid in DnaK was proposed to perturb the conformation of loop 5,6 in the peptide-binding domain, and when combined with a second mutation (G455D), the peptide off-rate increased (Buchberger *et al.,* 1999). Based on their positions (Figure 5), the *kar2-1* and *kar2-133* gene products may exhibit a similar kinetic defect; however, the contribution of single mutations, ATP, and/or Hsc70/DnaK cochaperones on peptide release was not examined in these other studies. Thus, it will be vital in future work to assess the peptide affinities and on- and off-rates for wild-type BiP and Kar2-1/133p in the presence and absence of each of the growing number of known BiP cochaperones, which include activators of ATP hydrolysis (e.g., Sec63p, Scj1p, and Jem1p) and nucleotide exchange factors (reviewed in Fewell *et al.,* 2001); for example, the Sls1p nucleotide exchange factor catalyzes nucleotide release from yeast BiP, and the introduction of the *kar2-1* and *kar2-133* alleles into *sls1*Δ yeast results in synthetic effects on cell growth (Kabani *et al.,* 2000b).

Another, nonmutually exclusive scenario is that the Kar2-1p– and Kar2-133p–mediated ERAD defects arise at least in part from defective "gating" of the translocation channel during retro-translocation. Experiments from Johnson and colleagues (Hamman *et al.,* 1998; Haigh and Johnson, 2002) indicate that BiP helps seal the translocation channel in the mammalian ER during translocation, but it is not clear how BiP facilitates channel reopening during retro-translocation (Johnson and Haigh, 2000). Therefore, it is formally possible that the Kar2p mutants are able to gate the translocon during protein import, but cannot do so during ERAD; this hypothesis is currently being examined. BiP-dependent gating might also require the participation of cochaperones, and the differential interaction between Kar2-1p and translocation/retro-translocation-specific factors might also explain why *kar2-1* yeast are selectively defective for ERAD.

Finally, as noted above, we have shown that increasing the amount of BiP in the ER does not facilitate ERAD in wild-type yeast but instead slows retro-translocation and degradation. In the *kar2-1* background, the introduction of wild-type BiP helps resolubilize pαF but ERAD efficiency is also reduced. These data may be explained by the fact that prevention of polypeptide aggregation requires only BiP binding, whereas ERAD requires both polypeptide binding and release, which might be regulated by cochaperones. PαF might have become bound to and trapped in the ER by exaggerated amounts of BiP (Figure 9), but the concentrations and/or activities of cochaperones required for release might not have risen in parallel. Consistent with our work, increasing the level of BiP slows the ERAD of mutant ribophorin in mammalian cells (deVirgilio *et al.,* 1999) and the concept that ERAD can proceed only after BiP release is well established (Knittler *et al.,* 1995; Beggah *et al.,* 1996; Skowronek *et al.,* 1998; Chillaron and Haas, 2000). It is possible that under conditions of ER stress, when the concentration of BiP rises via the UPR, ERAD efficiency may decline, but the solubility of putative ERAD substrates is maintained. In turn, if ER stress is removed and/or the level of BiP can return to that in the unstressed state, BiP-bound, aggregation-prone substrates may be given a "second-chance" to fold or may then be targeted for degradation. To test this hypothesis, it will be important in the future to correlate how different levels of ER stress affect the concentration of BiP, ERAD efficiency, and the solubility of ERAD substrates.

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