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BiP and Sec63p are required for both co- and posttranslational protein translocation into the yeast endoplasmic reticulum

(DnaJ/heat shock proteins/70-kDa heat shock protein)

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ABSTRACT **Two interacting heat shock cognate proteins in the lumen of the yeast endoplasmic reticulum (ER), Sec63p and BiP (Kar2p), are required for posttranslational translocation of yeast α-factor precursor *in vitro*. To investigate the role of these proteins in cotranslational translocation, we examined the import of invertase into wild-type, *sec63*, and *kar2* mutant yeast membranes. We found that Sec63p and Kar2p are necessary for both co- and posttranslational translocation in yeast. Several *kar2* mutants, one of which had normal ATPase activity, were defective in cotranslational translocation of invertase. We conclude that the requirement for BiP/Kar2p, which is not seen in a reaction reconstituted with pure mammalian membrane proteins [Görlich, D. & Rapoport, T. A. (1993) *Cell* 75, 615–630], is not due to a distinction between cotranslational translocation in mammalian cells and posttranslational translocation in yeast cells.**

Protein translocation into the endoplasmic reticulum (ER), the process by which a nascent polypeptide is transported across the lipid bilayer and into the lumen, can occur either during or after polypeptide synthesis. In cotranslational translocation in mammals, the emerging amino-terminal signal sequence of precursor proteins interacts with the signal-recognition particle (SRP), a ribonucleoprotein particle that arrests translation and targets the ribosome-linked nascent polypeptide chain to the SRP receptor at the ER membrane (reviewed in refs. 1 and 2). The signal sequence directs the insertion of the nascent chain into the ER membrane so that once translation resumes, the elongating polypeptide is threaded into the lumen of the ER. In posttranslational protein translocation, the nascent polypeptide is completely translated on soluble ribosomes before being translocated into the ER lumen. Cytosolic chaperones in yeast, in particular the 70-kDa heat shock cognate proteins (hsc70s), are required to keep the polypeptide in a translocation-competent form (3, 4). Precursor proteins with folded domains are unable to cross biological membranes (5). Translocating proteins also interact with lumenal chaperones that prevent premature protein folding and aggregation (6).

Protein translocation occurs both co- and posttranslationally in *Saccharomyces cerevisiae*. Mutations in the *S. cerevisiae* homologs of SRP, the SRP receptor, and the SRP RNA lead to the accumulation of untranslocated precursor proteins (7–11), and some precursor proteins will translocate into ER-derived microsomes *in vitro* only during translation (12–14). In contrast, other precursors can be posttranslationally translocated into microsomes *in vitro* (12–16), and Rothblatt *et al.* (17) demonstrated that one precursor was posttranslationally translocated *in vivo*. Surprisingly, disruption of the genes that encode the SRP and SRP receptor subunits is not lethal, although the resulting strains grow slower (7–11, 18). Assuming that the yeast SRP and SRP receptor homologs are required for cotranslational translocation as they are in mammals, the simplest interpretation of this result is that the posttranslational translocation pathway will also accommodate proteins that are usually imported cotranslationally.

A number of temperature-sensitive mutants that accumulate untranslocated protein precursors at the nonpermissive temperature have been isolated in yeast (reviewed in refs. 19 and 20). Four of these genes, known as *SEC61, SEC62, SEC63*, and *KAR2*, are essential. The predicted protein sequences of Sec63p and Kar2p (BiP) suggest that they are members of the family of molecular chaperones (21–23). Sec63p is an integral membrane protein in the ER and contains a lumenal segment that is 43% identical over 70 amino acids to the DnaJ protein from *Escherichia coli* (21, 24). Kar2p (22, 23) is a lumenal hsc70 that is homologous to mammalian BiP, a factor identified initially because it binds to incompletely assembled protein complexes (25, 26). Kar2p is also about 50% similar at the amino acid level to the *E. coli* hsc70, DnaK (23). During the initiation of phage λ DNA replication in *E. coli*, the DnaJ and DnaK proteins form a complex in which the ATPase activity of DnaK is stimulated by DnaJ (27). Both biochemical and genetic data demonstrate that Sec63p and Kar2p also interact (28, 29): a protein complex containing equimolar amounts of Sec63p, Kar2p, and two other Sec proteins has been isolated (28). However, in the complex prepared from *sec63-1* cells, which contains a point mutation in the DnaJ domain of *sec63* (30), Kar2p is unable to associate stably with Sec63-1p (28). Furthermore, when adenosine 5′-[γ-thio]triphosphate is present during the purification of the Sec63p complex, Kar2p dissociates, suggesting that both the intact DnaJ domain of Sec63p and hydrolyzable ATP are required for efficient association. The purified Sec63p–Kar2p complex restores translocation activity to reconstituted proteoliposomes that are prepared from membranes containing the *sec63-1* mutation (28). In addition, suppressors of the *sec63-1* temperature-sensitive mutation were isolated that map to the *KAR2* gene, and certain pairwise combinations of *sec63* and *kar2* mutant alleles display synthetic lethality—a genetic interaction that may indicate a physical association between two gene products (29). Thus, an ATP-regulatable DnaK–DnaJ pair required for protein translocation exists in the lumen of the yeast ER.

Görlich and Rapoport (31) reported the reconstitution of protein translocation using purified membrane proteins from the mammalian ER in which neither BiP nor a mammalian Sec63p homolog was required. The absence of BiP from this system was unexpected, given that others showed that undefined ER lumenal components are essential for translocation into the mammalian ER (32) and that Kar2p is necessary for protein translocation in yeast (19, 20). It is possible that the

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Abbreviations: ER, endoplasmic reticulum; SRP, signal-recognition particle; Inv, invertase; InvF, 31-kDa amino terminal fragment of Inv; ppαf, yeast prepro-α factor; 3gpαf, signal peptide-processed and triply glycosylated pro-α factor; hsc70, 70-kDa heat shock cognate protein.

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translocation machinery in yeast and mammals has diverged sufficiently so that Sec63p and BiP are no longer required for translocation in higher eukaryotes or that these molecules are required only for posttranslational translocation, because the mammalian translocation assay with reconstituted vesicles measures cotranslational translocation (31). In yeast cells, Kar2p is necessary for the translocation of all secretory proteins examined, including those that are translocated only cotranslationally *in vitro* (33). Conceivably, this requirement is imposed indirectly as a consequence of an inability to clear the available translocation channels of posttranslational cargo. In yeast microsomes, both Kar2p and Sec63p are required for posttranslational translocation (17, 34), although their role in *in vitro* cotranslational translocation has not been examined. To address this role and to test the possibility that the BiP-dependent cotranslational defect is indirect, we compared cotranslational protein translocation using yeast microsomes prepared from wild type, *sec63-1*, and various *kar2* mutant strains. We show that microsomes with these mutations are defective for cotranslational protein translocation even when they are isolated from cells grown at the permissive temperature.

MATERIALS AND METHODS

Yeast strains used were RSY151 (*Matα, leu2-3,112, pep4-3, ura3-52, sec63-1*), RSY156 (*MATα, leu2-3,112, pep4-3, ura3-52*) (17), RSY801 (*MAT***a**, *ade2-101, leu2-3,113, ura3-52*), RSY586 (*MATα, ade2-101, leu2-3,113, ura3-52, kar2-159*), RSY578 (*MATα, ade2-101, leu2-3,113, trp1-1, ura3-52, kar2-113*), and RSY579 (*MATα, ade2-101, leu2-3,113, trp1-1, ura3-52, kar2-203*) (34). Yeast were grown in YPD medium [2% Bacto-peptone/1% yeast extract (both from Difco)/2% dextrose] at the indicated temperatures and, where noted, were shifted to 37°C for 1.5 hr.

Microsomes were prepared and treated with micrococcal nuclease (Boehringer Mannheim) as described (35, 36). For both co- and posttranslational protein translocation assays, microsomes were first incubated in buffer [20 mM Hepes, pH 6.8/250 mM sorbitol/150 mM KOAc/5 mM $Mg(OAc)_2$] containing an ATP-regeneration mixture (36) at a final protein concentration of 1 mg of protein per ml (100-μl total volume) for 20 min at either 20° or 37°C. ATP was required during the preincubation to prevent microsome lysis (data not shown). The microsomes were collected by centrifugation in a Tomy (Tokyo) refrigerated Microfuge at 15,000 rpm for 10 min at 4°C and resuspended in buffer to a final protein concentration of 10 mg/ml. Posttranslational translocation reactions with yeast prepro-α factor (ppαf) were performed as described (36). For cotranslational protein translocation assays, mRNA was transcribed from either pG2SUC23 (corresponding to full-length invertase; Inv) or pG2SUC91 (corresponding to the 31-kDa amino-terminal fragment of invertase; InvF) by using phage SP6 polymerase as described by Hansen *et al.* (12, 15). Translocation reactions (30-μl total volume) contained 3 μl of the preincubated and washed microsomes, 4–6 μg of RNA, 15 μCi (555 kBq) of [^{35}S]methionine (Amersham), 10 μl of nuclease-treated gel-filtered yeast cytosol (35), 20 units of RNase inhibitor (Amersham), 10 μg of creatine phosphokinase, 10 μl of 3× translation buffer [66 mM Hepes, pH 7.4/75 mM creatine phosphokinase (Sigma)/2.25 mM ATP/300 μM GTP/120 μM amino acid mix without methionine (Fisher Scientific)/360 mM KOAc/6 mM $Mg(OAc)_2$/5.1 mM dithiothreitol] and diethylpyrocarbonate-treated water. After 1 hr at 20°C, reaction mixtures were placed on ice, divided in two, and either incubated with proteinase K (Sigma) at 0.5 mg/ml for 25 min or mixed with trichloroacetic acid to a final concentration of 20%. Attempts to measure cotranslational translocation with reconstituted vesicles prepared from solubilized yeast microsomes (36) or with cytosol prepared from the *sec65* strain that encodes the yeast SRP19 homolog (9) were unsuccessful. Samples were processed as described (36), analyzed by SDS/PAGE, and quantified by using a Molecular Dynamics PhosphorImager. The presence of translocated, glycosylated forms of Inv and InvF on SDS/PAGE was confirmed by analyzing parallel samples of concanavalin A-precipitated reaction products (data not shown). All values represent the mean of at least three independent experiments.

Kar2p from both wild-type (RSY801) and the *kar2-113* yeast strains was purified as described (36) except that glass-bead lysis was performed in the presence of 1 mM ATP and 0.5% Triton X-100 to improve protein yields. ATPase assays were conducted by the method of Flynn *et al.* (37).

RESULTS AND DISCUSSION

Kar2p is Required for Post- and Cotranslational Protein Translocation in Yeast. The assay for protein translocation into the yeast ER relies on the ability of a radiolabeled protein precursor to transit across the membrane and thus become inaccessible to exogenously added protease. The precursor of a yeast mating pheromone, ppαf, can be translocated posttranslationally *in vitro* (13, 15, 16), while the precursor of the secreted enzyme invertase (Inv) is translocated exclusively cotranslationally (ref. 12; J.L.B., unpublished observations). InvF can be posttranslationally imported, but it is imported more efficiently cotranslationally (ref. 12; J.L.B., unpublished observations). As ppαf enters the ER, its signal peptide is removed, and the polypeptide receives three core oligosaccharide moieties. The translocated species—signal peptide-processed and triply glycosylated pro-α factor (3gpαf), was resolved from ppαf by SDS/PAGE (see Fig. 1*A*, wild type) and was protected from protease in the absence of detergent (Fig. 1*A* wild type, lane 2). Translocated 3gpαf was degraded by trypsin when membrane integrity was lost upon the addition of 1% Triton X-100 (data not shown). Similarly, the translocation of secreted Inv results in the removal of its signal peptide, followed by addition of up to nine core oligosaccharides (38). These forms were resolved from the precursor molecule by SDS/PAGE (Fig. 1*B*, wild type). We chose these substrates to investigate whether mutations in *kar2* and *sec63* rendered microsomes defective for posttranslational and cotranslational protein translocation.

To address the role of Kar2p in protein translocation, we grew cells containing the *kar2-159* temperature-sensitive mutation at 20°C (permissive temperature) and then shifted them to 37°C (nonpermissive temperature) for 1.5 hr. Membranes were prepared and assayed for their ability to translocate ppαf, Inv, and InvF at 20°C. We found that the *kar2-159* microsomes were unable to translocate either ppαf posttranslationally or Inv and InvF cotranslationally (data not shown). This result corroborates previous studies showing that *kar2-159* microsomes and reconstituted vesicles were defective for the translocation of ppαf (34, 36). This is also in agreement with reports that the *kar2-159* strain accumulates both co- and posttranslationally translocated protein precursors at the nonpermissive temperature (33) and that depletion of Kar2p also results in the cytosolic accumulation of precursors (33, 39). Together, these results suggest that Kar2p is required for both co- and posttranslational protein translocation.

One hypothesis for the observed defect in Inv translocation in the *kar2-159* microsomes and cells (33, 39) assumes that initially only posttranslationally translocated precursors accumulate upon shifting the cells to the nonpermissive temperature. These partially translocated substrates then "jam" the common translocation complex or pore, leading to a translocation defect for all precursor proteins. To address this possibility, microsomes from *kar2-159* and the isogenic wild-type strains were prepared from cells grown at the permissive temperature. The microsomes were incubated at 20°C or 37°C,

collected by centrifugation, and assayed for the posttranslational translocation of ppαf and cotranslational translocation of Inv. Preincubation of the microsomes at the restrictive temperature was required because translation of Inv mRNA in yeast cytosol was inhibited at temperatures greater than 20°C (data not shown); thus, all translocation assays were conducted at 20°C. Because the microsomes used in these experiments were prepared from cells grown at the permissive temperature and were extensively washed (see *Materials and Methods*), they should not contain partially translocated protein precursors.

Fig. 1 and Table 1 show the results of preincubating wild-type and *kar2-159* microsomes prepared from cells grown at the permissive temperature and assaying the samples for co- and posttranslational translocation activity. As expected, microsomes from the *kar2-159* strain were defective for ppαf translocation (42% of wild-type activity), and this defect was exaggerated when the microsomes were preshifted to 37°C (6% of wild-type activity). The corresponding values for cotranslational translocation of Inv in the *kar2-159* microsomes were 84% and 17% with respect to wild-type membranes. We conclude that the temperature-sensitive defect in *kar2-159* that hinders translocation *in vivo* can also be observed *in vitro* and affects the translocation of both co- and posttranslationally translocated substrates.

We extended these observations to other temperature-sensitive mutations in the *kar2* gene. Microsomes prepared from the *kar2-113* and *kar2-203* mutant strains display a temperature-sensitive defect for ppαf translocation (34). As presented in Table 1, both the *kar2-113* and *kar2-203* microsomes showed a temperature-sensitive defect for ppαf translocation when assayed after either a 20°C or 37°C pretreatment. Similarly, the normalized level of translocation of Inv in the *kar2-113* microsomes decreased from 97% to 7% (20°C versus 37°C preshift) and in the *kar2-203* microsomes decreased from 113% to 57%. Therefore, three distinct mutant forms of Kar2p display various defects in cotranslational translocation.

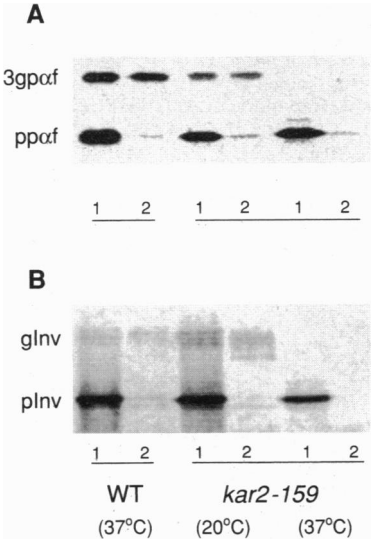

FIG. 1. *kar2-159* microsomes are temperature-sensitive for protein translocation *in vitro*. Wild-type (WT) and *kar2-159* cells were grown overnight at 20°C in YPD, and microsomes were prepared, preincubated at either 20°C or 37°C, and assayed for the translocation of either ppαf (*A*) or Inv (*B*) as described in *Materials and Methods*. Translocation efficiencies, determined as the percent of protease-protected and glycosylated product relative to the total amount of radiolabeled material in each reaction, were ≈45% and ≈20% for ppαf and Inv, respectively. Lanes: 1, aliquot of total translocation reaction; 2, protease-treated aliquot. gInv, Glycosylated Inv; pInv, preinvertase.

Table 1. ppαf and Inv translocation defects in microsomes containing the *sec63* and *kar2* mutations

	% translocation			
	ppαf		Inv	
Strain	20°C	37°C	20°C	37°C
Wild type	100	98	100	115
kar2-159	42	6	84	17
kar2-113	37	23	97	7
kar2-203	61	14	113	57
Wild type	100		100	
sec63-1	11		15	

Microsomes were prepared and assayed for either post- or cotranslational translocation of ppαf or Inv, respectively, as described in *Materials and Methods*. Values are expressed as percent translocation relative to the amount of protease-protected glycosylated product in the wild-type microsomes that were preshifted at 20°C and represent the means of three independent determinations.

The defects at 20°C for ppαf translocation in the Kar2p mutants were consistently greater than for Inv translocation (Table 1). This phenomenon may reflect unique requirements for Kar2p in co- versus posttranslational translocation (see below).

A Mutation in the *SEC63* Gene Compromises Post- and Cotranslational Protein Translocation. The protein encoded by the *SEC63* gene, Sec63p, associates with Kar2p when purified from detergent-solubilized yeast microsomes but fails to copurify with Kar2p when membranes are prepared from the *sec63-1* mutant strain (28). *sec63-1* microsomes and reconstituted vesicles are also defective for the posttranslational translocation of ppαf (17, 28). Because Sec63p and Kar2p are physically and possibly functionally associated, we assayed microsomes containing the *sec63-1* mutation for cotranslational protein translocation. *sec63-1* microsomes were only 11% as active for ppαf translocation and 15% as active for Inv translocation as the isogenic wild-type microsomes (see Table 1). The severity of the translocation defect did not increase when the microsomes were preincubated at 37°C (data not shown). Thus, both wild-type Sec63p and Kar2p are required for co- and posttranslational protein translocation in yeast.

Mutant Forms of Kar2p Contain Different Biochemical Defects. To better understand how mutations in *kar2* prevent protein translocation, we attempted to purify Kar2p and measure its ATP hydrolysis activity from both the wild-type and the *kar2* temperature-sensitive strains. Kar2p from the *kar2-203* and *kar2-159* mutants was unable to bind to ATP-agarose. Comparison of wild type and Kar2-159p binding to ATP-agarose by quantitative immunoblotting with iodinated protein A showed that only 9% of wild-type Kar2p flowed through the ATP-agarose column, whereas 77% of Kar2-159p was not retained on the column. The ATP-agarose elution profiles of Kar2-203p and Kar2-159p were qualitatively similar as determined by immunoblotting with enhanced chemiluminescence (Amersham; data not shown). In contrast, Kar2-113p bound to and was released from the column upon addition of ATP. Purified Kar2-113p had an ATPase activity of 1.3 nmol/min per mg of protein, while that of wild-type yeast Kar2p was 1.2 nmol/min per mg, measured at 37°C. These values are somewhat higher than the reported ATPase activity of mammalian BiP (0.33 nmol/min/mg) (37). We conclude that the translocation defect in the *kar2-113* microsomes is not due to aberrant ATPase activity. Instead, Kar2-113p may not associate with either precursor proteins or with Sec63p. The translocation defect in the *kar2-159* and *kar2-203* microsomes may relate to the inability of Kar2-159p and Kar2-203p to bind ATP. It is possible that the mutations in *kar2-159* and *kar2-203* cause a conformational or oligomeric change in the proteins that prevent their association with ATP-agarose or that they

are stably bound to a factor that occludes their ATP binding sites.

To determine whether the mutations in *kar2* affect protein stability *in vivo*, we prepared extracts from wild-type, *kar2-113*, *kar2-159*, and *kar2-203* cells and determined the relative amounts of Kar2p by quantitative immunoblotting with iodinated protein-A. Although Kar2-113p and Kar2-159p were present in nearly equal amounts as wild-type Kar2p (data not shown), the amount of Kar2-203p was significantly reduced (≈28% of wild-type levels). We conclude that the mutation in *kar2-203* renders the gene product less stable than wild-type Kar2p.

Model for Sec63p–Kar2p Function During Protein Translocation in Yeast. We suggested previously that Kar2p may act at two steps in the protein translocation cycle: at an early step, before the precursor peptide interacts with Sec61p (the putative translocation pore), and at a later stage, once the precursor becomes engaged by Sec61p but before translocation is complete (34). At an early stage in translocation, Kar2p might act in conjunction with Sec63p to help transfer the precursor molecule to the pore, to reprime the translocation complex after a round of translocation, or to activate the pore to begin protein translocation (20). This function of Kar2p would presumably be required for both co- and posttranslationally translocated substrates. The absence of a requirement for BiP in the reconstituted mammalian translocation reaction (31) may imply mechanistic differences between yeast and mammalian translocation, or it may reflect a failure of this purified system to reproduce faithfully multiple rounds of import by a single Sec61 complex. In contrast, Kar2p stimulates ppαf translocation when added to reconstituted vesicles containing the purified yeast translocation complex, of which Sec63p is one component (40).

In the second stage, Kar2p may pull or "reel-in" the translocating polypeptide and ensure that the process is unidirectional by sequential ATP-dependent binding, nucleotide hydrolysis, and dissociation from the nascent chain (41). The mitochondrial hsc70 has been proposed to function similarly (42–44). Kar2p may be required only in the first step of a cotranslational event because translation by the ribosome may be sufficient to promote unidirectional import.

An alternate interpretation of our data is that cotranslational protein translocation is not as tightly coupled in yeast as it is in mammals; thus, the translocation defects in the *sec63* and *kar2* strains are actually the result of posttranslational blocks. This explanation seems unlikely because microsomes prepared from the *sec62-1* strain translocate Inv and InvF normally, yet are severely compromised in their ability to translocate ppαf (35). This observation, together with the data presented here, shows that some components of the yeast translocation machinery are expendable for cotranslational translocation (Sec62p), while other factors (Kar2p and Sec63p) are not. We favor a model in which the Sec63p/Kar2p complex conveys both co- and posttranslational protein cargo from distinct receptor complexes (Sec62p for posttranslational proteins and SRP/SRP receptor for cotranslational substrates) to a common translocation channel, Sec61p (19, 20).

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