Interaction between BiP and Sec63p Is Required for the Completion of Protein Translocation into the ER of *Saccharomyces cerevisiae*

Susan K. Lyman and Randy Schekman
Department of Molecular and Cell Biology, Howard Hughes Medical Institute, University of California, Berkeley, California 94720

**Abstract.** To clarify the roles of Kar2p (BiP) and Sec63p in translocation across the ER membrane in *Saccharomyces cerevisiae*, we have utilized mutant alleles of the essential genes that encode these proteins: kar2-203 and sec63-1. Sanders et al. (Sanders, S. L., K. M. Whitfield, J. P. Vogel, M. D. Rose, and R. W. Schekman. 1992. *Cell.* 69:353–365) showed that the translocation defect of the kar2-203 mutant lies in the inability of the precursor protein to complete its transit across the membrane, suggesting that the luminal hsp70 homologue Kar2p (BiP) binds the transiting polypeptide in order to facilitate its passage through the pore. We now show that mutation of a conserved residue (A181→T) (Nelson, M. K., T. Kurihara, and P. Silver. 1993. *Genetics.* 134:159–173) in the luminal DnaJ box of Sec63p (sec63-1) results in an in vitro phenotype that mimics the precursor stalling defect of kar2-203. We demonstrate by several criteria that this phenotype results specifically from a defect in the luminal interaction between Sec63p and BiP: Neither a sec62-1 mutant nor a mutation in the cytosolically exposed domain of Sec63p causes precursor stalling, and interaction of the sec63-1 mutant with the membrane-bound components of the translocation apparatus is unimpaired. Additionally, dominant KAR2 suppressors of sec63-1 partially relieve the stalling defect. Thus, proper interaction between BiP and Sec63p is necessary to allow the precursor polypeptide to complete its transit across the membrane.
proteins interact. In an effort to modulate it may indicate that DnaJ-like proteins play a role in their ability to preferentially bind unfolded polypeptides and then hydrolyze ATP to release the bound peptide. ATPase activity is central to the function of hsp70s, the ability of DnaJ homologues to modulate may indicate that DnaJ-like proteins play regulatory roles in hsp70-mediated reactions such as translocation.

BiP (Kar2p) and Sec63p comprise the hsp70-DnaJ pair in the lumen of the ER. Genetic (Scidmore et al., 1993) and biochemical (Brodsky and Schekman, 1993) evidence indicates that these two proteins interact. In an effort to understand the dynamics of BiP and Sec63p function in protein translocation, we have examined a mutant of sec63 (sec63-I) (Rothblatt et al., 1989) that is unable to interact normally with BiP (Brodsky and Schekman, 1993). We show here that the sec63-I mutation, which maps to the J box of Sec63p (Nelson et al., 1993), is unable to support the completion of translocation. Interestingly, this precursor stalling phenotype was also previously observed in a translocation-defective mutant of BiP (Kar2-203) (Sanders et al., 1992). We demonstrate that the stalling phenotype of sec63-I is primarily due to a defective interaction between BiP and Sec63p, rather than to ancillary effects of the sec63-1 mutation, and also find that KAR2 suppressors of sec63-1 partially relieve the translocation defect of sec63-1 membranes. Since BiP interacts with Sec63p via the J box (Brodsky and Schekman, 1993), this suggests that successful completion of translocation is integrally linked to proper interaction between the hsp70 homologue BiP and its DnaJ cohort, Sec63p.

Materials and Methods

Yeast strains used were RSY801 (ura3-52, leu2-3-112, ade2-101, MATa); RSY579 (kar2-203, sec63-1, leu2-3-112, ade2-101, trplA1, MATa); RSY156 (ura3-52, leu2-3-112, pep4-3, MATa); RSY155 (sec63-1, ura3-52, leu2-3-112, ade2-1, pep4-3, +PMR397 [KAR2+URA4+]). RSY1151 (sec63-1, ura3-52, leu2-3-112, ade2-1, pep4-3 + PMR1676 [KAR2-6139, URA4+]); RSY1152 (sec63-1, ura3-52, leu2-3-112, ade2-1, pep4-3 + PMR1676 [KAR2-6139, URA4+]); RSY1153 (sec63-1, ura3-52, leu2-3-112, ade2-1, pep4-3 + PMR1676 [KAR2-6139, URA4+]), and RSY1154 (sec63-1, ura3-52, leu2-3-112, ade2-1, pep4-3 + PMR1650 [KAR2-6199, URA4+]). The pMR plasmids are URA3-centromere plasmids that carry either wild-type KAR2 or dominant KAR2 suppressors of sec63-1, as isolated by Scidmore et al. (1993).

Affinity-purified antibodies to Sec61p, Sec62p, and Sec63p were prepared as described in Stirling et al. (1992), Deshaies and Schekman (1990), and Feldheim et al. (1992), respectively.

Preparation of Yeast Microsomes

Yeast microsomes were prepared by a modification of the procedure described by Deshaies and Schekman (1989). Briefly, yeast were grown in YP (1% Bacto yeast extract, 2% Bacto peptone) + 2% dextrose to a final OD600 of 4. Wild-type strains were grown at 30°C; the sec and kar mutations were grown at 24°C. Cells were harvested at 4,000 g for 5 min at room temperature (RT), washed once in water, and resuspended to 50 OD units/ml in 0.7 M sorbitol, 75% YP, 0.5% dextrose, 10 mM Tris, pH 7.4, 5 mM DTT. To digest the cell wall and prepare spheroplasts, 40 units lyticase per OD unit was added and cells were incubated at 20°C for 30 min. Lyticase was prepared as in Shen et al. (1991). Spheroplasts were collected by centrifuging at 17,000 g; 10 min). The final microsome pellet was resuspended in B88 to 10 mg protein/ml as determined by Lowry assay and stored in aliquots at -70°C.

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S-labeled microsomes were prepared by glass bead-lysis of spheroplasts, as described in Feldheim et al. (1992).

Translocation

S-labeled pepF was transcribed and translated in vitro as described in Rothblatt and Meyer (1986). The translation product was purified on a
Q-Sepharose (Pharmacia Diagnostics Inc., Fairfield, NJ) column equili-
brated in Buffer A (8 M urea, 10 mM Heps, pH 7.4), washed with Buffer
A + 75 mM KOAc, and eluted with Buffer A + 200 mM KOAc. The eluted
fractions were stored in aliquots at -70°C.

Translocation reactions were carried out as in Brodsky et al. (1993) ex-
cept that 20 µg of microsomes were used per 20-µl reaction. 35S-ppaF was
diluted 1:20 into the translocation reaction, and urea was added to main-
tain a final concentration of 0.8 M. Translocation efficiency as determined
by the production of protease-protected, glycosylated ppF was assayed by
precipitation of the trypsin-treated translocation reactions with Con-
canavalin A Sepharose (Pharmacia). Completed reactions were solubi-

lized with 1% SDS and diluted with 1 ml Con A buffer (50 mM NaCl, 1%
Trion X-100, 20 mM Tris, pH 7.5, 2mM Na3H). Concanavalin A-Sepharose
(40 µl of a 20% suspension) was added, and after a 2-h RT incubation re-
actions were processed as described in Baker et al. (1988) and quantified by
scintillation counting. Percent translocation was defined as the ratio of
Concanavalin A-precipitated cpm (3gp~xF) to total ppF input into the re-
action.

Signal sequence cleavage was assessed by TCA precipitation of the
completed translocation reactions and resolution of the products on 10%
polyacrylamide gels as described by Brodsky et al. (1993).

Cross-linking and Immunoprecipitation

Cross-linking reactions contained 300 µg of microsomes, an ATP-regener-
ating system (1 mM ATP, 50 µM GDP-mannose, 40 mM creatine phos-
phate, 0.2 mg/ml creatine phosphokinase), ~300,000 cpm of 35S-ppaF, and
urea to 0.8 M final concentration in B88. 35S-labeled microsome reactions
consisted of membranes isolated from 5 OD units of cells plus the ATP-
regenerating system. Reactions were incubated at 20°C for 40 min, after
which cross-linking was initiated by the addition of DSP (Pierce Chem.
Co., Rockford, IL) to 0.5 mM, and reactions were processed as in Sanders
et al. (1992). For precursor stalling analysis, immunoprecipitations were
performed with 10 µg anti-Sec61p antibody and Sec61p-associated 35S-ppaF
was quantified by scintillation counting. For gel analysis of 35S-labeled
membranes, immunoprecipitations were carried out with 10 µg anti-
Sec61p antibody. The final pellets from these reactions were brought up in
Laemmli sample buffer + 5% BME, heated for 15 min at 65°C to reverse the
DSP cross-link, and resolved on 12.5% polyacrylamide gels.

Results

The Sec63-1 Mutation Results in Precursor Stalling

To assess the role of Bip and Sec63p in the translocation reaction, we used the sec63-1 mutant, which contains a conserved alanine to threonine change (A181→T) in the luminal DnaJ domain of Sec63p (Nelson et al., 1993). Bip interacts with Sec63p via the J box (Scidmore et al., 1992; Brodsky and Schekman, 1993), so this mutation is ex-
pected to uncouple Bip from its normal interaction with Sec63p, as shown by Brodsky and Schekman (1993). We examined the activity of sec63-1 membranes in vitro by analy-
alyzing translocation efficiency and by assessing the for-
mation of a stalled translocation intermediate. Upon entry into the ER lumen, the precursor protein ppaF undergoes signal sequence cleavage and is core glycosylated, result-
ing in the triply glycosylated luminal form 3gpaaF (Waters and Blobel, 1986a). The in vitro translocation assay traces the posttranslational import of in vitro-translated 35S-
labeled ppF into yeast microsomes by measuring the amount of ppF that is core-glycosylated and inaccessible to exogenously added protease (Rothblatt and Meyer, 1986; Waters and Blobel, 1986b). The precursor stalling assay assesses the extent to which 35S-ppaF is able to traverse the membrane by using cross-linking to stabilize the intermediate complex of pore and precursor that forms when luminal entry is blocked. Immunoprecipita-
tion with anti-Sec61p antibody isolates the stalled complex of pore and precursor. The extent of 35S-ppaF association with Sec61p in the mutant strains is indicative of the sever-
ity of the precursor stalling defect, whereas in wild-type membranes the association of ppF with the pore is tran-
sient, so significant cross-linking to Sec61p does not occur.

Sanders et al. (1992) previously used this assay to show that the translocation defect of kar2-203 lay in its inability to support the final phase of translocation, leaving the pre-
cursor stranded in the pore and unable to enter the lumen. Intriguingly, we find that the translocation-defective mu-
Gent see63-1 (Rothblatt et al., 1989) is also unable to com-
plete the transfer of precursor into the lumen. Fig. 1 shows the translocation and precursor stalling activities of mi-
crosomes derived from wt, sec63-1, or kar2-203 strains. Both kar2-203 and sec63-1 membranes are unable to pro-
cede past formation of a precursor-pore intermediate, as shown by the precursor stalling assay (Fig. 1 B). This block results in a severe translocation defect (Fig. 1 A). As a low level of nonspecific (i.e., ATP independent; data not shown) Sec61p-ppaF association occurs in wild-type mi-
crosomes, Sec61p-ppaF association in membranes derived from kar2-203 and sec63-1 strains is normalized to this level. Data in Fig. 1 B is the average of six assays in which the standard deviation was ~10%. The kar2-203 mutant consistently shows a twofold more severe stalling defect than does the sec63-1 mutant; a difference that will be addressed in the Discussion. Because lesions in kar2 and the J box of sec63 block translocation at an identical point, we propose that Bip and Sec63p cooperate in the final phase of translocation to deposit precursor into the lumen.

Figure 1. kar2-203 and sec63-1 mutants display both a transloca-
tion defect and a precursor stalling defect, while neither a mu-
tation in Sec62p nor a mutation in the cytosolically exposed domain of Sec63p results in precursor stalling. Reactions consisting of yeast microsomes, an ATP-regenerating system and the precursor protein 35S-ppaF were assayed for translocation efficiency (A) by Concanavalin A precipitation of the luminal, triply glyco-
sylated form of precursor (3gpaaF). The extent of stalled precursor in the reactions (B) was determined by cross-linking with DSP, immunoprecipitation with anti-Sec61p antibody, and scintillation counting of the Sec61p-ppaF complexes. Values are given as a ratio of Sec61p-ppaF cross-linking in the mutant membranes to the low level of cross-linking present in wild-type membranes (taken as 1×). In a standard reaction, ~300,000 cpm of 35S-ppaF (a saturating amount) was added; the stalled 35S-ppaF cross-linked to Sec61p in kar2-203 membranes typically comprised 6,000-8,000 cpm. Complete procedures are given in Materials and Methods.
Precursor Stalling Is Specific to the Lumenal Portion of Sec63p

Genetic data (Scidmore et al., 1993) suggests that BiP interacts with Sec63p through the J box: several mutant alleles of kar2 are synthetically lethal with the lumenal sec63-1 mutant, but not with cytoplasmic mutants of sec63. In support of this, Brodsky and Schekman (1993) have shown that in a wild-type strain BiP and Sec63p copurify in a complex with Sec71p and Sec72p; but BiP is released from the complex if the sec63-1 strain is used in the purification. Since the mutation in sec63-1 maps to a conserved residue in the luminal J box (Nelson et al., 1993), the most economical explanation for the precursor stalling phenotype of sec63-1 is that this mutation gives rise to a protein that is unable to interact productively with BiP, thus compromising the transfer of precursor into the lumen. It is, however, formally possible that the stalling phenotype is simply a generalized consequence of introducing lesions into the Sec63p molecule, or more basically, of interfering with a translocation component. To address this possibility, we used a mutation that maps to the cytosolically exposed domain of Sec63p (Klyce and McLaughlin, 1973; Sadler et al., 1989) as seen in Fig. 2. In vitro, this cytoplasmic mutant (sec63-108) displays neither a severe translocation defect nor precursor stalling (Fig. 1), suggesting that the luminal, rather than the cytosolic, portion of Sec63p is the critical region involved in the final phase of precursor transfer into the ER lumen. We additionally found that a mutation in sec62 (sec62-1), although defective for translocation (Fig. 1 A; Rothblatt et al., 1989), does not result in stalling (Fig. 1 B); implying that the inability to complete translocation is not simply the result of compromising the function of a part of the translocation apparatus.

Interaction of sec63-1 with a Translocation Subcomplex Is Unimpaired

Sec63p exists in a complex with four other components of the translocation apparatus (Sec61p, Sec62p, Sec71p, Sec72p). Cross-linking followed by denaturing immunoprecipitation with anti-Sec63p antibody preserves this five-member complex (Deshaiies et al., 1991). To examine the formation of the complex in the sec63-1 mutant, 35S-labeled membranes were prepared from either wild-type or sec63-1 strains. Cross-linking and immunoprecipitation with anti-Sec63p antibody (Fig. 3) yielded the expected complex, consisting of Sec63p, Sec61p, Sec71p, and Sec72p. Cross-linking and immunoprecipitation of the sec63-1 membranes (Fig. 3) gave a pattern identical to that of wild type. Sec62p is not seen in the anti-Sec63p immunoprecipitate because of either its low abundance or its low labeling efficiency (Feldheim et al., 1992), but anti-Sec62p immunoprecipitation yielded the intact complex in both wild type and sec63-1 membranes (data not shown). Since the interaction of the sec63-1 protein with the membrane-bound components of the translocation apparatus is unimpaired, this suggests that the precursor stalling defect of the sec63-1 membranes can be traced to the defect in BiP-Sec63p interaction that results from the sec63-1 J box mutation.

KAR2 Suppressors of sec63-1 Partially Relieve the Precursor Stalling Defect

Scidmore et al. (1993) have isolated dominant KAR2 suppressors of sec63-1 based on their ability to partially suppress the temperature sensitive growth phenotype of a sec63-1 strain. The suppressors are specific for the sec63-1 allele, as they do not mitigate the growth defects of muta-

[Figure 2. Model of the topology of Sec63p in the ER membrane and location of the sec63-1 and sec63-108 mutants. (Figure adapted from Nelson et al., 1993; Feldheim et al., 1992.)]

[Figure 3. The interaction of the sec63-1 protein with the membrane-bound components of the translocation apparatus is unimpaired. 35S-labeled microsomes were prepared from wild-type and sec63-1 strains to assess formation of a translocation subcomplex. Reactions containing labeled membranes isolated from 5 OD units of cells and an ATP-regenerating system were cross-linked with DSP and immunoprecipitated with anti-Sec63p antibody. The immunosolated complex was treated with reducing agent to reverse the cross-link, and products were resolved on 12.5% polyacrylamide gels. The asterisk marks the position of a band distinct from Sec61p that is precipitated nonspecifically in the absence of cross-linker.]
tions in the cytosolically exposed domain of Sec63p. These dominant KAR2 suppressors might be expected to alleviate the precursor stalling defect if they function by restoring proper interaction between BiP and Sec63p. Fig. 4 shows that when the plasmid-borne KAR2 suppressors are introduced into the sec63-1 strain, they reduce the severity of the stalling defect. The most potent suppressor in vitro, KAR2-6199, is also among the strongest of the suppressors of the sec63-1 translocation defect in vivo (Seidmore et al., 1993). KAR2-6199 restored sec63-1 translocation in vitro from 12.5% of wild-type activity to 51% of wild-type activity, and routinely reduced the severity of the stalling defect by 40–50%. The other three KAR2 suppressors relieved the translocation and stalling defects by lesser but still significant amounts (Fig. 4), while a control strain containing a wild-type KAR2 plasmid did not suppress the sec63-1 defect (data not shown). Interestingly, all of the KAR2 suppressor plasmids manifested a translocation defect to varying degrees when transformed into a wt background: wild-type strains harboring either KAR2-6199, KAR2-6139, KAR2-6116, or KAR2-699 showed 50, 76, 43, or 4% of wt translocation activity, respectively (data not shown). In a wt background, two of the suppressor plasmids (KAR2-699, KAR2-6116) showed no precursor stalling defect, while KAR2-6139 displayed a slight increase in precursor stalling (1.3× wt levels) and KAR2-6199 showed a somewhat more significant increase in stalling (1.9× wt levels; data not shown). The defects produced by the presence of the KAR2 suppressor plasmids in a wt background may be due either to nonproductive interaction of the Kar2p suppressor with wt Sec63p or with another translocation component, to the formation of poisoned mixed oligomers consisting of the suppressor Kar2p and the endogenous wt Kar2p, or to a combination of these effects.

The Stalled Precursor Is Not Signal Sequence Cleaved

To assess at what stage the stalled translocation intermediate is blocked, we determined whether or not the precursor ppαF was signal sequence cleaved in the kar2-203 and sec63-1 mutants. Upon the entry of ppαF into the lumen, two modifications normally occur: the NH₂-terminal signal sequence is cleaved and the protein is glycosylated at three sites in the pro region (Waters and Blobel, 1986a). The stalled intermediate may or may not protrude into the lumen, depending on at what stage the kar2-203 and sec63-1 mutants impose a translocation block. Fig. 5 A shows in vitro translocation of ppαF in microsomes derived from kar2-203 and sec63-1 strains. Comparison of the translocation product with standards of pro-alpha factor (paF) and the triply glycosylated form (3gpαF) demonstrates that ppαF is neither signal sequence-cleaved nor glycosylated in the mutant membranes. To directly examine the stalled ppαF that is associated with Sec61p in the mutant membranes, the Sec61p-ppαF complex was immunoisolated in vitro from cross-linked translocation reactions. Treatment with reducing agent reversed the cross-link, and products were resolved by SDS-polyacrylamide gel electrophoresis. No stalled ppαF signal was evident in immunoisolated reactions from wild-type membranes (data not shown). In immunoprecipitated reactions from sec63-1 and kar2-203 membranes (Fig. 5 B), as in the total translocation reactions, the precursor complexed with Sec61p is neither signal sequence cleaved nor glycosylated. Thus, the obstacle that the kar2-203 and sec63-1 mutants present to translocation leaves the precursor enveloped by the pore, inaccessible to lumenal processing.

Discussion

We have shown that a mutation in the conserved luminal J box of Sec63p (sec63-1) (Nelson et al., 1993) blocks translocation at the same point as was previously identified for kar2-203, a mutation in the NH₂-terminal ATPase domain (Vogel, 1993) of the hsp70 homologue BiP (Sanders et al., 1992): the precursor is engaged by the pore but cannot complete its membrane transit, so is subsequently left stranded in the pore, unable to enter the lumen. We have demonstrated by several criteria that this precursor

Figure 4. Dominant KAR2 suppressors of sec63-1 partially alleviate the translocation and precursor stalling defects of the sec63-1 mutation. Membranes derived from wild-type or sec63-1 strains, or strains containing the sec63-1 mutation plus the plasmid-borne KAR2 suppressor as indicated, were analyzed for translocation efficiency (A) and precursor stalling (B) as described in Methods and Materials.

Figure 5. The stalled precursor in kar2-203 and sec63-1 membranes is neither signal sequence cleaved nor glycosylated. (A) Translocation reactions containing wt, kar2-203 or sec63-1 membranes were assessed as either total reactions (1); trypsin-treated, to degrade untranslocated precursor (2); or Triton X-100 solubilized and trypsin-treated, to destroy membrane integrity and degrade microsomal contents (3). The positions of ppαF, paF (derived from tunicamycin-treated membranes; prepared as in Brodsky et al., 1993), and the doubly and triply glycosylated forms (2gpαF and 3gpαF) are shown to the left as controls. (B) ppαF-Sec61p complex was immunoisolated from cross-linked translocation reactions with anti-Sec61p antibody. The complex was treated with reducing agent to reverse the DSP cross-link and products were resolved on 10% acrylamide gels. The positions of ppαF and paF are shown on the left as controls.
stalling phenotype in sec63-1 is likely to result specifically and primarily from defective interaction between BiP and Sec63p; an association mediated by the lumenal J box of Sec63p. Neither the sec62-1 mutant nor a mutation in the cytosolically exposed domain of Sec63p causes precursor stalling, implying that the defect is not a generalized consequence of lesions in Sec63p or of interfering with a translocation component, but is specific to the lumenal domain of Sec63p. Interaction of the sec63-1 protein with the membrane-bound components of the translocation apparatus (Sec61p, Sec62p, Sec71p, Sec72p) is unimpaired, while Brodsky and Schekman (1993) have shown that this allele is unable to interact with BiP. Additionally, dominant KAR2 suppressors of sec63-1 partially relieve the in vitro translocation and precursor stalling defects associated with the sec63-1 mutant. We have also demonstrated that the signal sequence of the precursor ppαF is not cleaved in either kar2-203 or sec63-1 membranes, indicating that the block in translocation prevents the precursor from protruding into the lumen.

All of these observations converge on the conclusion that interaction between BiP and Sec63p is necessary for the precursor protein to completely traverse the membrane and to enter into the lumen. The notion that BiP and Sec63p may interact, or act at the same step in translocation, has previously been suggested by the demonstration of synthetic lethality between the kar2-203 and sec63-1 alleles by Scidmore et al. (1993). The fact that both the kar2-203 and sec63-1 mutants are blocked in the final phase of precursor transit suggests that BiP and Sec63p cooperate to provide the impetus to transfer the precursor from its site in the pore assembly across the membrane and into the lumen. A role for BiP in this capacity in yeast has previously been suggested by the evidence of Sanders et al. (1992) and Nguyen et al. (1991), and in mammalian cells by the observation that luminal proteins are involved in the completion of translocation (Nicolitta and Blobel, 1993). A similar role has been proposed for the luminal hsp70 Ssα1p in mitochondrial protein import (Kang et al., 1990; Gambill et al., 1993), and both BiP (Sanders et al., 1992) and Ssα1p (Scherer et al., 1990) bind a precursor protein artificially arrested in transit across the membrane.

The paradigm for hsp70 function originally suggested by Pelham (1988) proposes that hsp70s bind hydrophobic surfaces and then release from substrate concomitantly with a round of ATP hydrolysis. Several systems have provided experimental substantiation for this model for hsp70 proteins in general (for review see Gething and Blobel, 1992) and for BiP in particular (Flynn et al., 1989, 1991; Blond-Elguindi et al., 1993; Gaut and Henderson, 1993). In the framework of the translocation reaction, the hsp70 proteins provide a means of modulating the folding state of the precursor protein: cytosolic hsp70s may maintain the precursor protein in an extended, import-competent state (Verner and Schatz, 1988) by binding the polypeptide during translation. Similarly, luminal hsp70s could prevent incorrect folding interactions within and between translocating polypeptides by coating them until translocation was complete.

The association of hsp70s with substrate is regulated by the ability of the hsp70s to bind and hydrolyze ATP. For the hsp70 homologue DnaK (E. coli) and the cytosolic hsp70 Ssα1p (S. cerevisiae), this activity is in turn regulated by a DnaJ protein, which stimulates the low intrinsic ATPase activity of its hsp70 partner (Liberek et al., 1991; Cyr et al., 1992). It is possible that Sec63p also is able to regulate the ATPase activity of BiP, although this has not yet been shown. Brodsky and Schekman (1993) have suggested that in addition to a putative regulatory function, one role of the J domain of Sec63p may be to serve as a protein anchor to increase the effective concentration of the luminal hsp70 BiP by docking it at the translocation pore. The whole of Sec63p, however, undoubtedly functions as more than the molecular glue by which BiP is stuck to the translocation complex. Sanders et al. (1992) have shown that the sec63-1 mutation decreases the ability of an artificially generated membrane-spanning intermediate to interact with the translocation pore, implying that Sec63p functions at an early step in translocation in addition to the later step for which we provide evidence in this report.

Sec63p has a large cytosolic domain, three transmembrane segments, and a luminal loop containing the conserved J box (Feldheim et al., 1992); see Fig. 2. This topology provides an ideal means of linking luminal BiP function to that of the membranous translocon. Interaction of BiP with the luminal J box would induce a conformational change in Sec63p that could then be allosterically transmitted to other components of the translocon, as Sec63p exists in a complex with Sec61p, Sec62p, Sec71p, and Sec72p (Deshaies et al., 1991; Brodsky and Schekman, 1993). Such communication between BiP and Sec63p could have a bipartite function: early, in activating the translocation complex to receive precursor, and later, in facilitating the final phase of precursor transfer across the membrane. We have provided evidence for the latter role, but the nature of the early function of Sec63p is still unclear. The early role may be carried out in cooperation with BiP, as the data of Sanders et al. (1992) suggests that BiP also may work in both early and late phases of the translocation process. Other evidence (Müsch et al., 1992; Sanders et al., 1992) has shown that the interaction of artificially generated membrane-spanning intermediates with the pore is ATP dependent, and that this dependence does not reflect a cytosolic requirement (Müsch et al., 1992). The formation of the stalled intermediate in kar2-203 and sec63-1 strains is also ATP dependent (S. K. Lyman and R. Schekman, unpublished data). Since BiP and the cytosolic hsp70 Ssα1p are the only ATPases known to be involved in translocation, this further implicates the participation of BiP in an early phase of the process, possibly in the pore-gating function proposed by Crowley et al. (1994).

Our data show that the apparent precursor stalling defect of the sec63-1 mutant is less dramatic than that of the kar2-203 mutant, yet both are severely defective for translocation. We propose that the sec63-1 mutation may affect the early function of Sec63p as well as its later function, while the primary defect of the kar2-203 mutation may be in the later, rather than the early, role of BiP. Thus, in sec63-1 membranes, a reduced number of precursor molecules would be able to successfully dock with the pore, while those that are able to dock would still be unable to transfer into the lumen. If the kar2-203 mutant were primarily defective for the later step, then the majority of precursor molecules would be able to dock but would then...
be blocked at the luminal transition, resulting in greater net precursor stalling compared to the sec63-1 mutant.

How might BiP and Sec63p cooperate to transfer a secretory precursor from the pore complex into the lumen, and what are their roles in the larger context of the translocation process? Sanz and Meyer (1989) have demonstrated that translocation is comprised of both ATP-independent and ATP-dependent phases, and Misch et al. (1992) have extended the analysis by showing that while binding of precursor to Sec62p occurs in the absence of ATP, the primary interaction of precursor in the presence of ATP is with Sec61p. These and other observations may be integrated into a model (Fig. 6) in which the translocation process consists of three phases. (a) The ATP-independent binding of precursor to an assembly consisting of Sec62p, likely the putative signal sequence receptors Sec71p and Sec72p (Feldheim et al., 1993; Feldheim and Schekman, 1994), and possibly Sec63p. (b) The transfer of precursor from the signal sequence receptor complex to the pore in an ATP-dependent docking reaction likely effected by a conformational change in the translocon, mediated via BiP and Sec63p. (c) The ATP-dependent transit of precursor from the pore to the lumen in a reaction also mediated by BiP and Sec63p. Alternatively, as previously suggested (Brodsky and Schekman, 1994), one of the roles of BiP might be to function in the repriming of the acceptor complex to ready it for a second round of translocation. The basic tenets of BiP and Sec63p function in this three-step model have been detailed by Brodsky and Schekman (1993).

Sec63p in this scenario would function as a middleman between early and late events, transducing the influence of BiP on the translocon through a series of allosteric conformational changes. Early in translocation, BiP interaction with Sec63p could activate the complex, allowing the pore to receive the waiting precursor. In the latter part of the reaction, Sec63p may directly pass the precursor to BiP, which could then use the energy of ATP hydrolysis to reel the polypeptide into the lumen (reviewed in Brodsky and Schekman, 1994).

An important consequence of precursor transfer to BiP is that of translocation directionality. The binding of BiP to the transiting polypeptide may provide the energetic asymmetry necessary to prevent precursor backsliding and ensure that it emerges on the luminal side (reviewed in Brodsky and Schekman, 1994; Wickner, 1994). Evidence for the involvement of the mitochondrial hsp70 in providing this unidirectionality has been presented by Ungermann et al. (1994). A second crucial function of BiP may be to assist folding of precursor upon its entry into the lumen. Hsp70s may promote correct folding by the binding and regulated, ATP-dependent release of exposed hydrophobic surfaces, thus preventing nonspecific hydrophobic aggregation. Several studies have implicated BiP in the normal folding pathways of secretory proteins (Ng et al., 1989; Hammond and Helenius, 1994; Melnick et al., 1994), lending support to this notion.

The coordination of hsp70 and DnaJ function in BiP and Sec63p creates a protein machine that forges a critical link between distinct phases of translocation: the docking of precursor to the pore assembly and its ultimate transit across the membrane. The hsp70-DnaJ motif is mirrored in several other protein transport systems as well. In E. coli, DnaJ and Dnak function in export of secreted proteins (Wild et al., 1992), and the cytoplasmic hsp70-DnaJ pair Ssa1p and Ydj1p is involved in protein translocation in S. cerevisiae (Chirico et al., 1988; Deshaies et al., 1988; Cyr et al., 1992). Recent work on protein import in mitochondria has demonstrated that the mitochondrial hsp70 Ssc1p is physically and functionally coupled with MIM44, a peripheral protein of the mitochondrial inner membrane that has a short stretch (18 amino acids) of similarity to the conserved J box (Rassow et al., 1994). This small region of DnaJ homology could function to target the matrix hsp70 to the membrane-bound import apparatus, upon which the hsp70-MIM44 complex might act as a molecular ratchet to mediate transfer of the precursor protein across the mitochondrial membrane and into the matrix (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994). Although the region of MIM44 similarity to DnaJ is slight, the pairing of hsp70 and DnaJ function in mitochondrial import provides an analogy to that of ER translocation and may represent an evolutionarily conserved mechanism. The hsp70-DnaJ interaction may be tailored to suit the needs of a specific system: In some cases, the DnaJ counterpart may regulate hsp70 function via ATPase stimulation; in others it may target hsp70 to certain substrates or serve as an anchor to localize soluble hsp70 to a membranous protein assembly (Cyr et al., 1994). Our data suggests that the conjunction of Sec63p and BiP in ER translocation may play multiple roles, both early and late, in translocation. Sec63p, in addition to anchoring BiP, may also act in the
regulated release of transiting polypeptides from BiP by stimulating its ATPase activity. Another intriguing possibility is that Sec63p may act as a relay or trigger for BiP to carry out its putative pore-gating function. The cytosolically exposed domain of Sec63p may act as a sensor that allosterically transduces a signal to alert BiP that a precursor is awaiting entrance to the pore, upon which an ATP-mediated conformational change originating from BiP in the lumen might induce the pore to open and allow the precursor to successfully transit the ER membrane.

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