A Sec63p-BiP Complex from Yeast Is Required for Protein Translocation in a Reconstituted Proteoliposome

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Abstract. Reconstituted proteoliposomes derived from solubilized yeast microsomes are able to translocate a secreted yeast mating pheromone precursor (Brodsky, J. L., S. Hamamoto, D. Feldheim, and R. Schekman. 1993. *J. Cell Biol.* 120:95–107). Reconstituted proteoliposomes prepared from strains with mutations in the *SEC63* or *KAR2* genes are defective for translocation; the *kar2* defect can be overcome by the addition of purified BiP (encoded by the *KAR2* gene). We now show that addition of BiP to wild-type reconstituted vesicles increases their translocation efficiency threefold. To identify other ER components that are required for translocation, we purified a microsomal membrane protein complex that contains Sec63p. We found that the complex also includes BiP, Sec66p (gp31.5), and Sec67p (p23). The Sec63p complex restores translocation activity to reconstituted vesicles that are prepared from a *sec63-1* strain, or from cells in which the *SEC66* or *SEC67* genes are disrupted. BiP dissociates from the complex when the purification is performed in the presence of ATPγS or when the starting membranes are from yeast containing the *sec63-1* mutation. We conclude that the purified Sec63p complex is active and required for protein translocation, and that the association of BiP with the complex may be regulated in vivo.

TRANSLOCATION of secretory proteins into the lumen of the ER is facilitated by cytosolic, lumenal, and ER membrane proteins (for review see Nunnari and Walter, 1992; Rapoport, 1992; Sanders and Schekman, 1992). In the yeast *Saccharomyces cerevisiae*, these factors were identified through a combination of genetic and biochemical methods. Genes that encode three of the ER membrane proteins were isolated using a genetic selection designed to discover temperature-sensitive mutations that impair the translocation of secreted polypeptides into the ER (Deshaies and Schekman, 1987; Toyn et al., 1988; Rothblatt et al., 1989; Deshaies and Schekman, 1990; Feldheim et al., 1992; Stirling et al., 1992). These genes, *SEC61*, *SEC62*, and *SEC63*, are essential and mutations in them display synthetic lethality, a result which suggested that the protein products may associate physically (Rothblatt et al., 1989). Indeed, when yeast microsomes were solubilized in detergent, treated with a cross-linking reagent, and incubated with an antibody to either Sec62p or sec63p, the three gene products coimmunoprecipitated (Deshaies et al., 1991). Two additional polypeptides were identified in the immunoprecipitate. One protein of molecular mass 31.5 kD is an integral membrane glycoprotein. The corresponding gene, *SEC66*, is not essential but yeast strains deleted for the gene are temperature-sensitive for growth and display translocation defects at both the permissive and non-permissive temperatures (Feldheim et al., 1993; Kurihara and Silver, 1993). The other polypeptide (p23 encoded by *SEC67*) is not essential for growth at any temperature, but nevertheless is important in the translocation of selected secretory protein precursors (Feldheim, D., and R. Schekman, unpublished data).

Sequence analysis of the *SEC61* and *SEC63* genes has yielded clues about their functions in protein translocation. Sec61p, a 54-kD protein, is predicted to span the ER membrane 8–10 times and contains conserved sequences that are found in proteins required for translocation in other species (Görlich et al., 1992; Rapoport, 1992; Stirling et al., 1992). One of these homologues, SecY, is thought to be the permease or channel through which translocating proteins pass in the inner membrane of *E. coli* (Joly and Wickner, 1993). Cross-linking studies have demonstrated that Sec61p in yeast, and a homologous protein in mammalian ER, contact translocating precursor polypeptides (Müsch et al., 1992; Sanders et al., 1992; Görlich et al., 1992). Sec63p is a 68-kD polypeptide that contains three membrane-spanning segments and harbors a sequence that is 43% identical over 73 amino acids to the dnaJ protein from *E. coli* (Sadler et al., 1989). Determination of the membrane topology of Sec63p verified that this region faces the ER lumen (Feldheim et al., 1992). During DNA replication of λ phage in *E. coli*, dnaJ interacts with the bacterial hsc70 homologue, dnaK, and stimulates the ATPase activity of dnaK (for review see Ang et al., 1991). In yeast, an hsc70 is located in the ER lumen,

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ideally situated to interact with the dnaJ domain of Sec63p. This hsc70, termed BiP or Kar2p, was cloned independently by two laboratories based on its sequence similarity to mammalian BiP and because mutations in the *KAR2* gene rendered cells defective for karyogamy, or nuclear fusion (Normington et al., 1989; Rose et al., 1989). Certain mutations in *KAR2* also displayed translocation defects, suggesting that BiP may be directly associated with the translocation complex (Vogel et al., 1990). This hypothesis is supported by the identification of *sec63-1* suppressor mutations that map to the *KAR2* gene (Scidmore et al., 1993).

To identify the function of these Sec gene products and other proteins required for translocation, it was necessary to reconstitute the translocation reaction. We solubilized yeast microsomes and introduced the proteins into soybean phospholipid vesicles (Brodsky et al., 1993). The resulting proteoliposomes translocated a yeast mating pheromone, prepro-α-factor (ppαF),[1] in an ATP-dependent reaction. When membranes from a translocation-defective *kar2* strain were used to prepare reconstituted vesicles, translocation was deficient. The *kar2* defect was remedied by the introduction of purified wild-type BiP into the vesicles. This result suggested that the reconstitution assay could assess other active components of the translocation machinery.

In this report, we demonstrate that a protein complex that contains Sec63p restores translocation activity to reconstituted vesicles prepared from *sec63-1* membranes. The complex was purified using conventional chromatographic methods and includes Sec63p, BiP, Sec66p (gp 31.5), and Sec67p (p23). Our data suggests that BiP physically associates with Sec63p in the ER, and that this interaction is regulated by ATP hydrolysis. We propose a cycle of BiP binding to the Sec63p complex and ATP hydrolysis linked to translocation.

Materials and Methods

Materials

Yeast strains used in this study were: RSY156 (*leu2-3, -112, ura3-52, pep4-3, MATα*), RSY151 (*sec63-1, leu2-3, -112, ura3-52, pep4-3, MATα*) (Rothblatt et al., 1989), YPH499 (*ura3-52, lys2-801, ade2-101, trpl-Δ63, his3-Δ200, leu2-Δ1, MATα*), and YPH500 (same as YPH499 except *MATα*) (Sikorski and Hieter, 1989). The gp31.5 disrupted strain was made by replacing two-thirds of the *SEC67* gene, including the transmembrane segment of the protein, with the *LEU2* gene in YPH500 (Feldheim et al., 1993). The p23 disrupted strain was made by gene replacement of the middle 50 amino acids of the Sec67 protein with His3 in YPH400 (Feldheim, D., and R. Schekman, unpublished data). All strains were grown in YPD (1% Bacto yeast extract, 2% Baco peptone, 2% dextrose) at 23°C unless otherwise indicated.

Antibodies to Sec63p and Sec66p have been described (Feldheim et al., 1992, 1993). We prepared an antiserum against Kar2p by injecting rabbits with a TrpE-Kar2p fusion protein (Rose et al., 1989). An antiserum against Sec67p was made by D. Feldheim (this laboratory) using a 19-amino acid peptide identical to the COOH terminus of the protein.

BiP/Kar2p and Ssalp were purified as previously reported (Brodsky et al., 1993). A bacterially expressed variant of BiP was also used and yielded identical results in our assays (data not shown). The variant contained six histidine residues at the NH_2 terminus of the mature protein and was purified using Nickel-NTA-agarose chromatography (Qiagen; Chatsworth, CA). The variant gene was constructed and the protein was kindly supplied by Joe Vogel (Rose Laboratory, Princeton University).

Purification of the Sec63p Complex

Yeast microsomes were prepared as previously described (Brodsky et al., 1993) based on an earlier protocol (Rothblatt and Meyer, 1986). All subsequent steps were performed at 4°C. Generally, 12 mg of microsomal protein were added to solubilization buffer (100 mM KP_i, pH 7, 500 mM KOAc, 10 mM DTT, 20% (vol/vol) glycerol) to a final concentration of 1 mg/ml. Sonicated azolectin at 50 mg/ml (45% phosphatidylcholine; Avanti Polar Lipids, Alabaster, AL) in 10 mM KP_i, pH 7.2, 1 mM β-mercaptoethanol, was next added to the microsome solution to yield a lipid to protein ratio of 3:1 (wt/wt). Finally, Ultrol grade β-octyl-D-glucopyranoside (OG) (Calbiochem, San Diego, CA) in water at a concentration of 10% was layered onto the mixture such that the final concentration of OG after mixing was 1.2%. The suspension was agitated on the highest setting using a Vortex mixer for 5 s and placed on ice for 15–30 min. To remove any insoluble material, we centrifuged the solution at 100,000 *g* for 30 min at 4°C. The supernatant fraction was loaded onto a 5-ml DEAE-Sepharose Fast Flow column (Pharmacia LKB Biotechnology, Piscataway, NJ) preequilibrated in solubilization buffer that also included 1.2% OG (Sigma Chem. Co., St. Louis, MO). The column was washed with 4 vol of buffer at a flow rate of 0.3 ml/min, and a 12-ml linear gradient from 0.5 M KOAc (solubilization buffer/OG) to 1.5 M KOAc (in the same buffer) was run through the column. Sec63p immunoreactivity eluted at a KOAc concentration of ~0.8 M, as measured on a CDM 80 conductivity meter (Radiometer, Copenhagen, DK).

The fractions containing Sec63p (2–3 ml) were concentrated to 0.5 ml with a centricon-30 microconcentrator (Amicon, Beverly, MA) and the protein was loaded onto a 22-ml Superose-6 column connected to a Pharmacia LKB FPLC. The column was preequilibrated with 100 mM KP_i, pH 7, 150 mM KOAc, 10 mM DTT, 20% (vol/vol) glycerol, 1.2% OG, and 3 mg/ml of azolectin (same as above), and eluted at 0.15 ml/min. The fractions demonstrating Sec63p immunoreactivity (14–15 ml) were loaded onto a 1-ml hydroxylapatite (HAP) column (BioRad Labs., Hercules, CA) preequilibrated in the Superose-6 buffer without lipid. The column was washed at 0.1 ml/min with 5 ml of buffer containing 200 mM KP_i before a linear gradient from 200 mM KP_i to 500 mM KP_i (same buffer plus an additional 300 mM KP_i) was applied. The Sec63p complex eluted at ~300 mM KP_i, determined as above by measuring the conductivity of an aliquot of the fraction in glass-distilled water.

Reconstitution and Rescue Assays

Reconstitution reactions using wild-type, *sec63-1*, and *sec66* or *sec67* null strains were performed as previously described (Brodsky et al., 1993). Briefly, microsomes were solubilized and centrifuged as described above, and the supernatant fraction was dialyzed against detergent-free buffer for 15–17 h. The dialysate was mixed with preformed, centrifuged liposomes at a lipid to protein ratio (wt/wt) of 100:1. The mixture was frozen in a dry ice-acetone bath, thawed, and centrifuged at 100,000 *g* for 30 min at 4°C. The supernatant was carefully removed and the soft, yellow-white pellet was redissolved in the residual buffer. This material was used directly in a translocation assay (see below). The translocation activity of the vesicles was stable for up to 4 h if kept on ice.

To determine the activity of the Sec63p complex, we performed a reconstitution as described below except that aliquots of the column fractions to be assayed were added directly to the centrifuged supernatant before dialysis. When necessary, liposomes were added at this time to maintain the lipid to protein ratio at 3:1 (wt/wt). Routinely, 0.32 ml of cleared microsomes were used in each dialysis (about 300 μg of protein), with up to 50 μg of added protein from the assayed fraction. Sec63p reconstitution activity was highest if the complex was purified within 2 d of solubilization and without freezing the samples between column steps. For other purposes, column fractions were frozen in liquid nitrogen and stored at −70°C.

General Methods

Translocation reactions with either microsomes or reconstituted proteoliposomes were performed as previously described (Brodsky et al., 1993) using ^{35}S-ppαF, an ATP-regenerating system (or 1 mM ATPγS for the no ATP control), and buffer 88 (20 mM Hepes, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc); yeast cytosol at a final concentration of 5 mg/ml was also included when the reconstituted vesicles were assayed. At the completion of a reaction, aliquots were either directly precipitated with 20% TCA (15 min on ice and then centrifuged in a microcentrifuge for 10 min

1. *Abbreviations used in this paper*: HAP, hydroxylapatite; ppαF, prepro-α-factor.

at 4°C), or treated with trypsin, or trypsin and Triton X-100 as before (Brodsky et al., 1993). SDS-PAGE (10%) was used to resolve the products and the results were visualized and quantified on a Molecular Dynamics PhosphorImager (Sunnyvale, CA). Net translocation was defined as the amount of protease-protected, processed label (pαF in reconstitution assays or triply-glycosylated pαF in the microsome-based assay) observed in the presence of trypsin, minus the residual signal in the detergent and protease-treated controls. Translocation efficiency was defined as the amount of net translocation divided by the signal in the total (TCA-precipitated) reaction.

Fractions from DEAE-Sepharose, Superose-6, and HAP columns were analyzed by 12.5% SDS-PAGE either directly after mixing aliquots with sample buffer (Laemmli, 1970), or after precipitating the proteins with 20% TCA (45 min on ice, followed by a 15-min centrifugation in a refrigerated microcentrifuge). Gels were either stained with silver or used to transfer the proteins to nitrocellulose for immunoblotting with polyclonal antibodies (Towbin et al., 1979). Visualization of the antibody-antigen complexes was achieved using goat anti-rabbit horseradish peroxidase-conjugated antibody and the enhanced chemiluminescence (ECL) kit (Amersham Life Sciences, Little Chalfort, Buckinghamshire, GB).

Protein quantitation was performed by TCA-precipitating proteins onto nitrocellulose by vacuum filtration and staining the blots with amido black. The stain was eluted and quantified with a spectrophotometer at a wavelength of 630 nm. Protein determinations (using BSA as the standard) were linear from 0–12 μg of protein in this assay. This procedure is similar to one in which proteins adsorbed onto a filter are stained with Coomassie brilliant blue G (Minamide and Bamburg, 1990).

Results

Addition of BiP Increases the Translocation Activity of Reconstituted Vesicles

Reconstituted proteoliposomes prepared from solubilized wild-type yeast microsomes can translocate radiolabeled ppαF in an ATP-dependent reaction, and convert ppαF to its signal peptide cleaved form, pαF (Brodsky et al., 1993). An example of this reaction is shown in Fig. 1. A portion of pαF is protected from trypsin digestion when the reaction is performed in the presence, but not the absence of ATP (compare lane 2, "−BiP" and "+ATP" to lane 2, "−BiP" and "−ATP"). Also as previously observed, some ppαF is resistant to protease digestion even in the presence of detergent (Brodsky et al., 1993). This material probably is aggregated ppαF.

The efficiency of the translocation reaction in reconstituted vesicles is between 5–10%, whereas the translocation efficiency of microsomes is ~50%. We attribute this discrepancy to either irreversible protein denaturation that occurs during solubilization, unproductive reassociation or reversed orientation of the translocation apparatus in the proteoliposome, or loss of one or more essential factors from the ER that are involved in translocation. A likely candidate for the last of these possibilities is the ER lumenal hsc70, BiP (Vogel et al., 1990). We showed previously that when BiP was added to solubilized *kar2* membranes, the *kar2* translocation defect in the reconstituted vesicles was corrected (Brodsky et al., 1993). In these experiments BiP was added to 1% of the total protein concentration. However, BiP is estimated to represent 5–10% of the total ER (Gething and Sambrook, 1992). Approximately 50% of yeast BiP is reconstituted into proteoliposomes while greater than 75% of Sec63p associates with the vesicles as determined by immunoblot analysis of insoluble and soluble microsomal extracts and sucrose gradient purification of the reconstituted vesicles (data not shown; Brodsky et al., 1993). Therefore, we tested the effect of reconstituting a wild-type proteoliposome with additional pure BiP. When 10% of the total protein in the reconstituted vesicles consisted of supplementary BiP, the translocation efficiency increased to ~30% (see Fig. 1, lane 2, "+BiP" and "+ATP"). Greater amounts of BiP failed to increase the translocation efficiency further, and neither BSA nor a yeast cytosolic hsc70, Ssalp, present as 10% of the total protein, raised the translocation efficiency beyond that of untreated, wild-type reconstituted vesicles (data not shown). Further control experiments showed that supplementary BiP did not restore translocation to proteoliposomes reconstituted from *sec63-1* mutant membranes (not shown), nor was supplemental BiP effective if it was added after the proteoliposomes were formed (Brodsky et al., 1993).

Purification of the Sec63p Complex

Reconstituted vesicles prepared from *sec63-1* membranes displayed a translocation defect (Brodsky et al., 1993). We used this deficiency to develop a biochemical complementation assay to allow the isolation of functional Sec63p. Wild-type yeast microsomes were solubilized as in the normal reconstitution and the soluble proteins were fractionated in the presence of 1.2% octylglucoside and 20% (vol/vol)

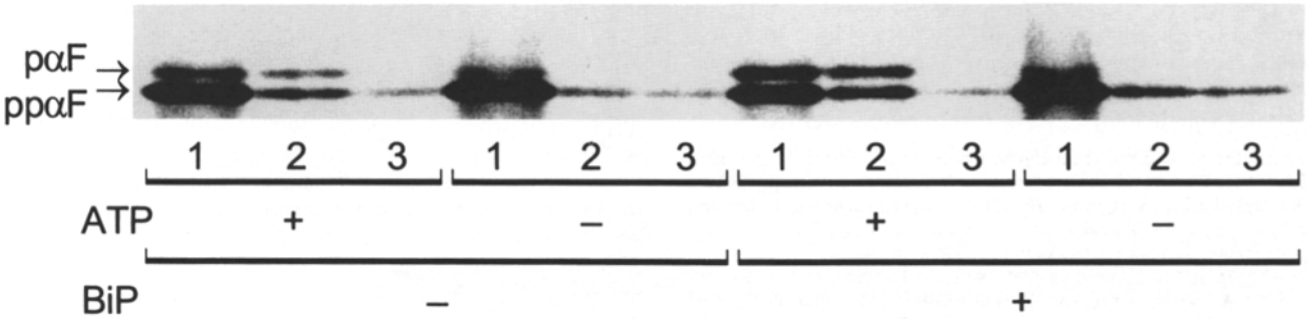

Figure 1. Supplementary BiP increases the translocation efficiency of reconstituted vesicles. Proteoliposomes were prepared from wild-type microsomes either in the absence or presence of purified yeast BiP (added to 10% of total protein), as indicated, and were assayed for the translocation of pre-proα factor. Translocation reactions contained either an ATP-regenerating system ("+ATP") or 1 mM ATPγS ("−ATP"). Aliquots from the reaction were precipitated (lane *1*), treated with protease before precipitation (lane *2*), or treated with protease and detergent before precipitation (lane *3*), and analyzed by 10% SDS-PAGE (see Materials and Methods for experimental details). *ppαF*, pre-proα factor; *pαF*, proα factor.

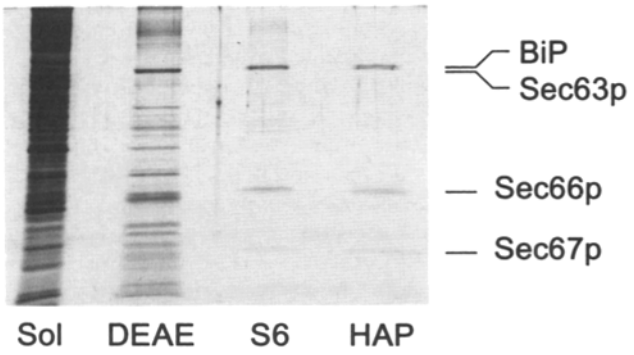

Figure 2. Purification of the Sec63p complex. Fractions that contained Sec63p immunoreactivity after each step in the purification were resolved by SDS-PAGE and the gel was stained with silver. *Sol*, detergent-solubilized and centrifuged membranes; *DEAE*, DEAE-Sepharose; *S6*, Superose-6; *HAP*, hydroxylapatite. Approximate molecular masses of the proteins are: *BiP*, 70 kD; *Sec63p*, 68 kD; *Sec67p*, 31.5 kD; *Sec66p*, 23 kD.

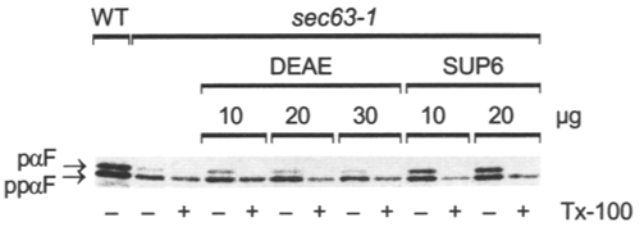

Figure 3. The Sec63p complex restores translocation activity to *sec63-1* reconstituted vesicles. Wild-type or *sec63-1* reconstituted vesicles were prepared in the absence or presence of either 10, 20, or 30 μg of protein from the DEAE-Sepharose column or Superose-6 column. The fractions used in this experiment contained the Sec63p complex, as determined by immunoblot analysis. The reconstituted vesicles were assayed for the translocation of ppαF, and all samples were treated with protease in either the presence or absence of 1% Triton X-100, as indicated in the figure.

glycerol (see Materials and Methods). By following Sec63p immunoreactivity with an anti-Sec63p fusion protein antibody (Feldheim et al., 1992), we observed that Sec63p and many other proteins remained bound to DEAE-Sepharose until high concentrations of KOAc (0.8 M) were reached (see Fig. 2, "DEAE"). A pool of the DEAE-Sepharose fraction containing Sec63p was next filtered on a Superose-6 column. Sec63p (a 68-kD protein) eluted coincident with a 440,000 molecular weight standard (ferritin). Other proteins in this fraction included three prominent polypeptides of 70, 31, and 23 kD (Fig. 2, S6). The 31- and 23-kD proteins coincided with two polypeptides previously seen as cross-linking partners to Sec61p, Sec62p, and Sec63p (Deshaies et al., 1991). The genes that encode the 31- and 23-kD proteins have recently been cloned and sequenced, and antibodies to the proteins have been raised (Feldheim et al., 1993; Feldheim, D., and R. Schekman, unpublished data). Because disruption of these genes results in translocation defects in vivo, we consider these genuine SEC genes and refer to the corresponding proteins as Sec66p (gp31.5) and Sec67p (p23) (Feldheim et al., 1993; Feldheim, D., and R. Schekman, unpublished data). Using the anti-Sec66p and anti-Sec67p antisera, we verified that the two polypeptides that coeluted with Sec63p are gp31.5 and p23. BiP, which migrates along with Sec63p on an SDS-PAGE, was detected by Kar2p immunoblot in the Sec63p-enriched fractions of the Superose-6 eluate. BiP also appeared in nearly every fraction that eluted from both the DEAE-Sepharose and Superose-6 columns.

To test whether any or all of these proteins were members of a bone fide protein complex, we analyzed the Sec63p fraction from Superose-6 by velocity centrifugation in glycerol. The fraction was diluted to 10% (wt/vol) glycerol, loaded onto a 12-ml 10–25% (wt/vol) glycerol gradient that also contained 1% OG, and centrifuged at 150,000 *g* for 24 h at 4°C. The peak Sec63p immunoreactivity sedimented at ~9.4S (relative molecular mass of 200 kD) with BSA (68 kD, 4.6S), aldolase (158 kD, 7.3S), and catalase (232 kD, 11.4S) used as standards in a parallel gradient (Waters et al., 1986). All of the Sec66p and Sec67p comigrated with Sec63p, as did a portion of the BiP that was loaded on the gradient (data not shown).

We also analyzed the Sec63p Superose-6 fraction by HAP chromatography. Sec63p immunoreactivity eluted on a 0.2–0.5 M linear phosphate gradient at 0.3 M phosphate, along with BiP, Sec66p, and Sec67p (Fig. 2 for the HAP peak fraction, and Fig. 4, "Wild Type" for the elution profile). Repetition of the HAP chromatography on Sec63p samples diluted to 0.1 M phosphate showed continued coelution of the four proteins at 0.3 M phosphate. Unlike the first HAP column, no BiP was present in the flow-through fraction of the repetition (Fig. 4, "Wild Type", BiP profile, wash lanes for the first column profile). Thus a fraction of the BiP in the lysate is stably and specifically associated with Sec63p, Sec66p, or Sec67p. Quantitative immunoblot analysis with radioiodinated Protein A revealed that 0.3% of total BiP copurified along with the complex. Approximately 3% of total Sec63p was recovered in this fraction. Thus, if all of the Sec63p is bound to BiP, ~10% of the total BiP is in the complex. In a control experiment, pure BiP eluted from the HAP column throughout the salt gradient used to isolate Sec63p. SDS-PAGE and densitometry of the Coomassie blue–stained proteins purified through HAP show that BiP and Sec63p are represented at a ratio of 1:1.4. Given the glycerol gradient estimate of 200 kD, the complex may contain one copy of each of the four proteins.

We assayed the ability of the Sec63p complex to restore translocation activity to *sec63-1* reconstituted vesicles. Protein from the Sec63p-containing fractions from DEAE-Sepharose and Superose-6 was added to solubilized and centrifuged *sec63-1* membranes, and reconstituted proteoliposomes were formed (see Materials and Methods). As shown in Fig. 3, addition of increasing amounts of the Superose-6 fraction restored translocation activity to the *sec63-1* vesicles. In this experiment, *sec63-1* vesicles were only 10% as active as wild-type proteoliposomes. When 20 μg of the Superose-6-purified complex was added to the *sec63-1* reconstitution, translocation activity increased to 50% of the wild-type level. The formation of protease-protected pαF was dependent on ATP in the reaction, and the complex purified from *sec63-1* membranes did not restore activity (data not shown).

The DEAE-Sepharose fraction from wild-type membranes partially rescued the *sec63-1* defect when 10 μg of protein was added (Fig. 3). Surprisingly, addition of more protein decreased the amount of translocation that was re-

Table I. Purification of the Sec63p Complex

Purification step	Protein	Specific activity (*U/mg*)	Total activity (*Units*)	Recovery
Solubilized extract	12 mg	0.84	10	100%
DEAE	0.36 mg	8.8	3.2	32%
Superose-6	7.0 μg	49	0.34	3.4%
Hydroxylapatite	2.0 μg	41	0.08	0.8%

Purification table for the Sec63p complex from solublilized yeast microsomes. One unit of translocation activity is defined as the equivalent of 100% of wild-type translocation activity restored to *sec63-1* reconstituted vesicles by a fraction from the Sec63p complex purification. In each assay, the amount of protein that restored activity to *sec63-1* reconstituted vesicles was in the linear range for the rescue of the *sec63-1* defect. Because the rescue of the *sec63-1* defect by the DEAE-Sepharose fraction is non-linear (see text for details), the activity for this fraction was calculated using 10 μg protein (see Fig. 3). All other activities were calculated using from 20 to 50 μg of protein.

stored to the *sec63-1* proteoliposomes (Fig. 3, 20 and 30 μg, DEAE). It is possible that an inhibitory factor is present in the extract and is enriched along with Sec63p in the DEAE eluate.

Addition of the wild-type Sec63p complex from the HAP column restored activity to the *sec63-1* reconstituted vesicles (see Table I). The specific activity of the HAP-purified complex was slightly lower than the complex from Superose-6. Activity may have decayed during the additional 6 h required to run the column and analyze the fractions. Sec63p activity in the Superose-6 fractions was 58-fold enriched with respect to the solubilized and centrifuged membrane extract.

The Nature of the BiP-Sec63p Interaction

We next examined the effect of ATP and the *sec63-1* mutation on the integrity of the complex. BiP is an ATPase and is 50% identical to the dnaK protein from *E. coli* (Normington et al., 1989; Rose et al., 1989). Sec63p contains a region that is similar to the *E. coli* dnaJ protein (Sadler et al., 1989), and dnaK and dnaJ are known to interact (Ang et al., 1991). We reasoned that the *sec63-1* mutation could affect the stability of BiP in the complex, because the mutation converts a conserved alanine in the dnaJ region to a threonine (Nelson et al., 1993). *sec63-1* cells were grown at the permissive temperature, microsomes were prepared, and the Sec63p complex was purified (see Materials and Methods). In the last purification step (hydroxylapatite) very little BiP eluted coincident with Sec63-1p, and instead a small amount eluted at a higher concentration of salt (Fig. 4, "*sec63-1*"). This was in contrast to the HAP fraction of the complex from isogenic wild-type microsomes, where BiP coeluted with Sec63p, Sec66p, and Sec67p (Fig. 4, "Wild Type"). The simplest interpretation of this result is that the mutation in the dnaJ domain of Sec63p reduces its affinity for BiP and renders the complex unstable to conventional chromatographic procedures.

We considered the possibility that ATP hydrolysis might regulate the binding of BiP to the Sec63p complex and that ATP or a non-hydrolyzable analog of ATP might lock BiP in a conformation that could no longer bind to Sec63p. However, the Sec63p complex remained intact when the purification was conducted in the presence of ATP and Mg^{2+}, perhaps because the ATP is hydrolyzed during the column elutions. In contrast, when the purification was carried

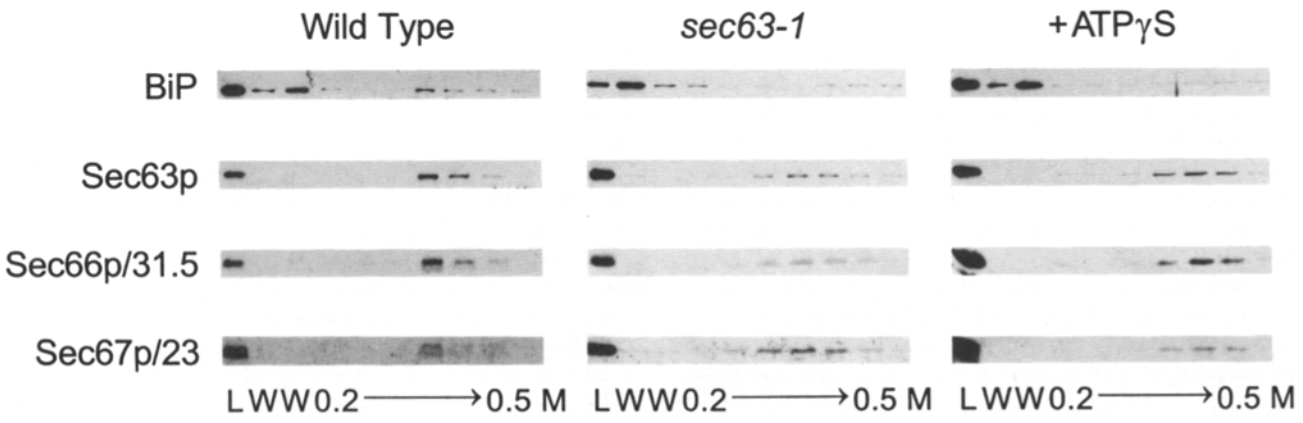

Figure 4. Purification of the Sec63p complex by HAP column chromatography. The Sec63p complex was purified from either wild-type or *sec63-1* microsomes using ion exchange and gel filtration chromatography as described in the Materials and Methods. The complex was then analyzed by HAP column chromatography and fractions were immunoblotted for the presence of BiP, Sec63p, Sec66p, and Sec67p. "+ATPγS" denotes that the Sec63p complex was purified in the presence of 1 mM ATP and 1 mM $MgCl_2$ during the DEAE-Sepharose and Superose-6 columns, and then in the presence of 0.5 mM ATPγS and 1 mM $MgCl_2$ during the HAP column. The results presented in this figure have been obtained in at least three independent experiments. The chromatographic spreading of Sec66p and Sec67p immunoreactive species in the wild-type purification was not due to protein heterogeneity because silver stain analysis of the Sec63p complex revealed unique electrophoretic forms of these proteins (see Fig. 2). *L*, loaded sample on the column; *W*, eluate from the 0.2 M KP_i column wash; *0.2–0.5 M*, fractions from a 0.2–0.5 M KP_i linear gradient applied to the column.

through two column steps (DEAE-Sepharose and Superose-6) in the presence of ATP and Mg^{2+}, and then chromatographed on HAP equilibrated and run in 0.5 mM ATPγS and 1 mM $MgCl_2$, BiP was resolved from the remaining members of the complex (Fig. 4, "+ATPγS"). We conclude that ATP may cause the dissociation of BiP from Sec63p.

Functional Replacement of Sec66p and Sec67p

Translocation defects in vivo have been observed in strains containing gene disruptions of *SEC66* and *SEC67* (Feldheim et al., 1993; Kurihara and Silver, 1993; Feldheim, D., and R. Schekman, unpublished data). We examined the translocation activity of membranes and proteoliposomes prepared from the mutant strains to assay the activity of the Sec63p complex in regard to Sec66p and Sec67p. The translocation activity of the *sec66* and *sec67* microsomes was compared to their respective isogenic wild-type membranes in reactions conducted at 20°C or 37°C for 40 min. Each 60 μl incubation contained 2 μl of microsomes (at 10 mg protein/ml), an amount that was in the linear range for translocation activity. The results, presented in Fig. 5 *A*, showed that *sec67* was only marginally defective for translocation of ppαF at 20°C (73% of wild type) while the *sec66* membranes were 50% as active as wild type. With both *sec66* and *sec67* microsomes, there was a slight temperature-sensitive defect when the translocation reactions were performed at 37°C (Fig. 5 *A*). Microsomes prepared from *sec66* mutant cells that had been incubated at the non-permissive temperature (37°C) for 30 min were no more defective in translocation.

Next, reconstituted vesicles were prepared from wild-type, *sec66* and *sec67* strains grown at 23°C. The translocation activities of the *sec66* and *sec67* proteoliposomes were about one-third of wild-type levels (Fig. 5 *B*, no addition). To determine whether the translocation activities of the *sec66* and *sec67* vesicles could be restored, we supplemented proteoliposome reconstitutions with wild-type Sec63p complex purified by DEAE-Sepharose and Superose-6 chromatography (see Materials and Methods and Fig. 2). As controls, the complex was also purified from the *sec66* and *sec67* strains. The Sec63p-containing fractions from the Superose-6 column prepared from these mutant strains included only BiP and other minor contaminating proteins; neither Sec66p nor Sec67p chromatographed in these fractions (data not shown). In accordance with these results, Feldheim et al. (1993) have shown that Sec67p cannot be cross-linked to the Sec63p complex in a solubilized lysate of the *sec66* deletion mutant strain. Wild-type Sec63p complex added during reconstitutions of mutant membranes restored translocation activity to ~70% of normal (Fig. 5 *B*). In contrast, defective complex isolated from the mutants failed to restore activity to reconstituted liposomes.

Discussion

We have isolated a protein complex from the yeast ER that contains Sec63p, BiP/Kar2p, Sec66p, and Sec67p. Mutations and/or disruptions in the genes for each of these factors result in translocation defects both in vivo and in vitro (Rothblatt et al., 1989; Vogel et al., 1990; Sanders et al., 1992; Feldheim et al., 1993; Kurihara and Silver, 1993; Feldheim, D., and R. Schekman, unpublished data). We have shown that reconstituted vesicles prepared from *sec63-1*, *sec66*, and *sec67* strains are defective for precursor protein translocation. Incorporation of the Sec63p complex into reconstituted proteoliposomes from each of these strains restores translocation activity. We conclude that the Sec63p complex is essential for ppαF translocation in proteoliposomes.

Cross-linking and immunoprecipitation experiments led us to suggest that a larger translocase complex exists in native membranes that includes Sec62p and at least a subset of the pool of Sec61p molecules as well as the Sec proteins described in this report (Deshaies et al., 1991). Even without treatment with a cross-linking reagent, a complex including Sec62p and Sec63p could be immunoprecipitated from detergent solubilized membranes. However, the purification steps developed for the isolation described in this report result in complete resolution of Sec62p and Sec61p from the Sec63p complex. Both proteins are removed in the very first step; chromatography on DEAE cellulose. Reconstitution of translocation with proteoliposomes formed from a mixture of detergent solubilized *sec63-1* membranes and pure Sec63p complex almost certainly requires the pool of Sec61p and Sec62p provided by the mutant membrane fraction. Reconstitution of the yeast translocation system with all pure components according to this view will require the isolation of functional forms of Sec61p and Sec62p.

Previously, we reported that BiP is required in the lumen to reconstitute translocation functional proteoliposomes (Brodsky et al., 1993). Our reconstitution procedure allows 10–25% of exogenous BiP to become enclosed within protease-inaccessible vesicles (Brodsky et al., 1993). Endogenous BiP also is incorporated, but the low efficiency of translocation (10% of the ppαF is translocated and signal peptide-processed) may be limited by the incomplete recapture of BiP during proteoliposome formation. We now find that addition of supplemental BiP to the detergent soluble fraction allows the formation of proteoliposomes that are significantly more efficient (~30%) in the translocation of ppαF. This stimulation is BiP selective because cytosolic hsc70, which can be incorporated into proteoliposomes (Brodsky et al., 1993), will not satisfy this requirement. Moreover, supplemented BiP does not bypass the normal requirement for wild-type Sec63p.

We also observed that addition of 10 μg of the Sec63p complex fails to restore translocation activity to reconstituted vesicles that contain the *kar2-159* mutation (data not shown). For comparison, when 1.6 μg of pure BiP is added to *kar2-159* reconstituted proteoliposomes, translocated activity is restored to ~80% of the wild-type level (Brodsky et al., 1993). These results suggest that the *kar2-159* defect is rescued by BiP that is not associated with the Sec63p complex.

The association of BiP with the complex is mediated by interaction with Sec63p. HAP column chromatography resolves BiP from the other members of the complex when the purification is conducted with a soluble fraction prepared from *sec63-1* membranes. The point mutation in *sec63-1* is in an invariant residue of the dnaJ-like domain of Sec63p (Nelson et al., 1993). An interaction between BiP and Sec63p was first proposed when the dnaJ homology was uncovered (Sadler et al., 1989), and was based on the interaction between dnaJ and dnaK (an hsc70 and BiP homolog;

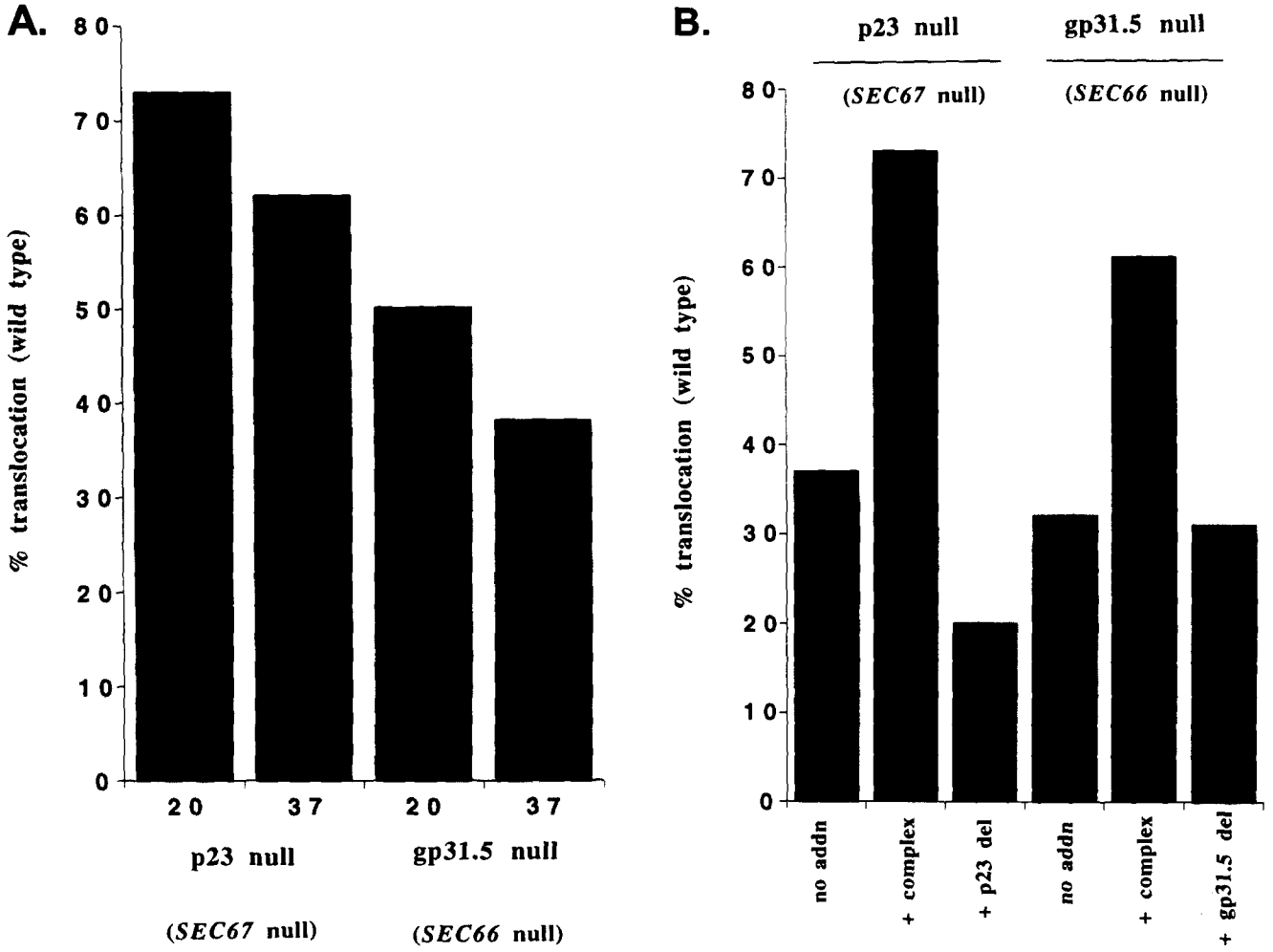

Figure 5. *sec66* and *sec67* microsomes and reconstituted vesicles are defective for translocation: the defect in the *sec66* and *sec67* reconstituted vesicles is remedied by the addition of the Sec63p complex. Microsomes (*A*) and reconstituted vesicles (*B*) were prepared and assayed for translocation activity as described in the Results and Materials and Methods. Translocation assays with the microsomes were performed at either 20° or 37°C (as indicated), while the reconstituted vesicles were assayed at 20°C. Translocation activity is presented as a percentage of that obtained with the isogenic wild-type membranes. As indicated in *B*, the proteoliposomes were untreated ("no addn"), or were supplemented with either the wild-type Sec63p complex (purified using the DEAE-Sepharose and Superose-6 columns), or the Sec63p complex from the *sec66* and *sec67* strains. The reconstituted vesicles (300 μg of total protein) included 0.6 μg of the Sec63p complex in the rescue assays; greater amounts of the complex failed to restore translocation activity further in the *sec66* and *sec67* reconstituted vesicles (data not shown). Data represent the means of at least two independent experiments.

Rose et al., 1989) during λ phage DNA replication in *E. coli*. Topological analysis of Sec63p verified that the dnaJ loop of the Sec63p faces the ER lumen, the compartment in which BiP is located (Feldheim et al., 1992). Genetic evidence also suggests that *KAR2* and *SEC63* interact (Scidmore et al., 1993). The results presented here strongly suggest that the two proteins physically associate.

It is a formal possibility, however, that BiP associates with another member of the complex, such as Sec66p or Sec67p, and that the *sec63-1* mutation destabilizes this association, which in turn releases BiP. However, Sec67p contains neither a signal sequence nor a transmembrane segment, and is therefore likely to be cytosolic (Feldheim, D., and R. Schekman, unpublished data), while only 15% of Sec66p is contained in the lumen of the ER (Feldheim et al., 1993). Overall, very little or none of these proteins is available to contact a protein in the ER lumen.

We conclude that the *sec63-1* defect in vivo is due to a labile Sec63p-BiP interaction. This lability is reflected in the observation that BiP was resolved from the *sec63-1* mutant complex even though neither the cells nor the membranes were exposed to the mutant non-permissive temperature. A weak interaction in the mutant complex may have been disrupted by the high phosphate concentration used during the elution of the proteins from HAP. A more sensitive method to analyze Sec63p-BiP association in native membranes would address this possibility.

Is the association of BiP with Sec63p regulated in the lumen of the ER in wild-type cells? The presence of ATPγS in the final purification step caused BiP to resolve from Sec63p. ATP, which is present in the ER lumen and is required for translocation (Mayinger and Meyer, 1993), may serve to regulate a cyclic association of BiP and Sec63p. BiP possesses a weak ATPase activity (Tokunaga et al., 1992),

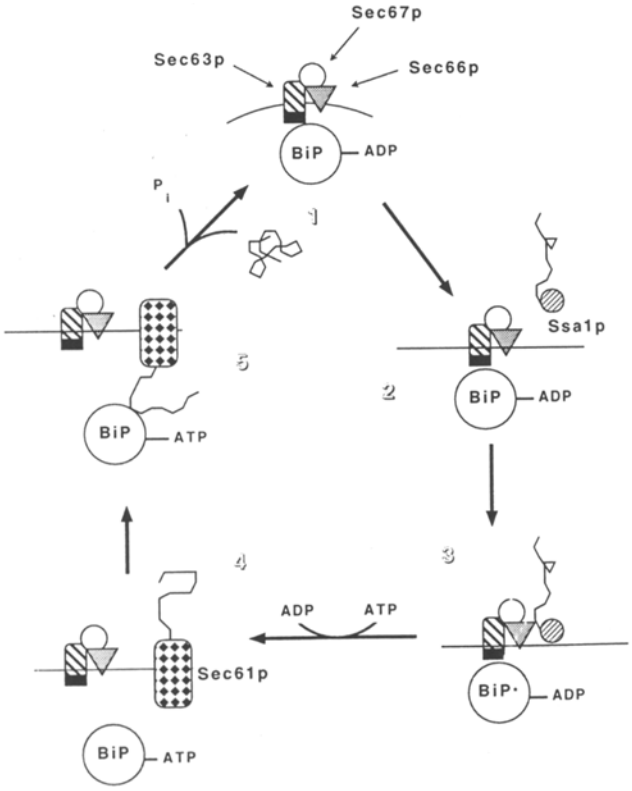

Figure 6. A model for the Sec63p complex function in protein translocation.

that may be influenced by interaction with Sec63p and other lumenal proteins.

Fig. 6 shows a model that summarizes our hypotheses on the regulation of BiP in the yeast ER. Stage 1 shows the Sec63p complex with BiP bound to ADP. BiP is assumed to be in the complex in an ADP-bound form because it is released when the complex is purified in the presence of ATPγS (Fig. 4). During or after translation, a secretory protein associates with cytosolic hsc70 (Ssa proteins) that maintains the polypeptide in a translocation competent conformation (stage 2; Deshaies et al., 1988; Chirico et al., 1988). Translocation is initiated by transfer of the polypeptide to the cytosolic face of the ER membrane where an initial interaction may occur with the Sec63p complex (stage 3), or with SRP receptor in the case of SRP-mediated translocation events (Nunnari and Waler, 1992). We propose that the interaction of the polypeptide with the complex facilitates a conformational change in Sec63p that is transduced across the membrane. As a result BiP is induced to exchange ADP for ATP (BiP* in stage 3) and is freed from the Sec63p complex (stage 4). This is analogous to the behavior of receptor-coupled trimeric G-proteins (for review see Gilman, 1987), where binding of ligand to the receptor releases the α-subunit of the G-protein from the membrane and causes the subunit to exchange GDP for GTP. The release of ADP and binding of ATP by BiP may require a nucleotide dissociation stimulator in the ER lumen (stage 3 to stage 4). The GrpE protein in *E. coli* catalyzes nucleotide exchange in dnaK (Ang et al., 1991). Although anticipated, a yeast homolog of GrpE that acts on BiP has not been found. In the absence of ATP, however, precursor proteins are unable to associate with Sec61p, the putative translocation pore (Müsch et al., 1992; Sanders et al., 1992). This may be explained by a failure to activate BiP (stage 3 to 4). Upon dissociation of BiP-ATP from the complex, the nascent chain may be transferred to Sec61p.

Another role for BiP involves direct interaction with the nascent secretory polypeptide in the ER lumen (stage 5, Sanders et al., 1992). Here, BiP may serve to facilitate refolding of the emerging polypeptide and to drive the translocation reaction forward by cycles of binding and dissociation to peptide domains coupled to ATP hydrolysis (Flynn et al., 1989). The hydrolysis of ATP by BiP may be facilitated by a transient interaction of BiP-ATP with the dnaJ domain of Sec63p. By analogy, the *E. coli* dnaJ protein stimulates ATP hydrolysis by dnaK (Ang et al., 1991). BiP-ADP may be recruited to the Sec63p complex at multiple points during or only after the final ATP hydrolysis event releases fully translocated polypeptide. It is possible, however, that the dnaJ and dnaK motifs have been conserved in Sec63p and BiP, respectively, merely to facilitate protein–protein interaction. In this case, Sec63p would not stimulate ATPase activity in BiP, and the hydrolysis of ATP by BiP and release of peptide occur either because of the intrinsic ATPase activity of BiP, or because of an as yet unidentified dnaJ-like activity in the ER lumen. Finally, because BiP associates poorly with the *sec63-1* complex, the *sec63-1* mutation would retard the transition from stage 5 to stage 1 (Fig. 6), which could result in a defect in the cyclic association and dissociation of BiP with the translocating polypeptide, or in a failure to create an activated Sec63p complex required to recruit the next secretory polypeptide from the cytosol. Certain aspects of this model can now be tested using ppαF and the purified complex from wild type, *sec63-1*, and ATPγS-treated membranes.

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Note Added in Proof: Dr. Neil Green (Department of Microbiology and Immunology, Vanderbilt University School of Medicine) recently determined that the *SEC66* and *SEC71* genes, and probably the *SEC67* and *SEC72* genes are identical. *SEC71* and *SEC72* were previously isolated as mutations that are unable to translocate a fusion protein into the yeast ER membrane (Green, N., H. Fang, and P. Walter. 1992. *J. Cell Biol.* 116: 597–604).

Our demonstration that BiP dissociates from the Sec63p complex on a hydroxylapatite column in the presence of ATPγS and a potassium phosphate gradient is in complete agreement with the recent results of Palleros, D., K. Reid, L. Shi, W. Welch, and A. Fink. 1993. *Nature (Lond.).* 365:664–666 who showed that ATPγS and K^+ are sufficient to release bound polypeptides from Hsp70s.

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