# Reconstitution of Protein Translocation from Solubilized Yeast Membranes Reveals Topologically Distinct Roles for BiP and Cytosolic Hsc70

**Jeffrey L. Brodsky, Susan Hamamoto, David Feldheim, and Randy Schekman**

Department of Molecular and Cell Biology, Howard Hughes Medical Institute, University of California, Berkeley, California 94720

*Abstract.* We reconstituted prepro-α-factor translocation and signal peptide processing using a yeast microsomal detergent soluble fraction formed into vesicles with soybean phospholipids. Reconstituted translocation required ATP, and was deficient when *sec63* and *kar2* (BiP) mutant cells were used as a source of membranes. Normal translocation was observed with vesicles reconstituted from a mixture of pure wild-type yeast BiP and a soluble fraction of *kar2* mutant membranes. Two other heat-shock cognate (hsc)70 homologs, yeast cytosolic hsc70 (Ssalp) and *E. coli* dnaK protein did not replace BiP. Conversely, BiP was not active under conditions where translocation into native ER vesicles required cytosolic hsc70. We conclude that cytosolic hsc70 and BiP serve noninterchangeable roles in polypeptide translocation, possibly because distinct, asymmetrically oriented membrane proteins are required to recruit each protein to opposing surfaces of the ER membrane.

THE precise mechanism whereby a charged, polar secretory polypeptide traverses the hydrophobic environment of the ER lipid bilayer remains poorly understood. It is now generally believed that proteins exist to facilitate this process. To identify some of these factors, our group (Deshaies and Schekman, 1987; Rothblatt et al., 1989; Stirling et al., 1992) and others (Toyn et al., 1988; Sadler et al., 1989; Green et al., 1992) have used the yeast *Saccharomyces cerevisiae* to screen for mutants that accumulate preproteins in the cytosol. Three of these temperature-sensitive mutants were designated *sec61*, *sec62*, and *sec63*. The products of these genes have been identified as integral membrane proteins of the ER (Deshaies and Schekman, 1987; Deshaies and Schekman, 1989; Rothblatt et al., 1989; Sadler et al., 1989; Feldheim et al., 1992). *SEC61*, *SEC62*, and *SEC63* have also been shown to interact genetically (Rothblatt et al., 1989), and the three proteins form a complex together with two other as yet unidentified proteins of 31.5 and 23 kD (Deshaies et al., 1991).

These Sec proteins are intimately associated with translocating proteins. A translocation-trapped intermediate form of the yeast mating factor precursor, prepro-α-factor (ppαF),[1] may be chemically cross-linked to Sec61p, and to a lesser extent, Sec62p (Müsch et al., 1992; Sanders et al., 1992). Cross-linking to Sec62p was ATP inhibited, while association with Sec61p was ATP dependent (Müsch et al., 1992). Mutations in *SEC62* or *SEC63* significantly reduced the degree of cross-linking to Sec61p (Sanders et al., 1992).

Translocating ppαF also associates with the heat-shock cognate protein (hsc70) homologue, BiP, in the ER lumen (Sanders et al., 1992). Earlier studies demonstrated a requirement for BiP with the discovery that mutations in its gene (*KAR2*) caused the accumulation of a variety of untranslocated secretory preproteins (Vogel et al., 1990). Intriguingly, Sec63p has a polypeptide domain facing the ER lumen that is 42% identical to the dnaJ protein from *Escherichia coli* (Sadler et al., 1989; Feldheim et al., 1992). Because Kar2p is 50% identical to the dnaK protein from *E. coli* (Rose et al., 1989), and the dnaK and dnaJ proteins interact during bacteriophage replication (Yamamoto et al., 1987), it is tempting to speculate that Sec63p and Kar2p also interact. Exactly how Kar2p facilitates translocation remains unknown. A principal function of hsc's may be to prevent protein aggregation, or to disassociate protein–protein interactions (Pelham, 1986). For example, mammalian BiP preferentially binds hydrophobic peptides (Flynn et al., 1991), and peptide binding stimulates ATP hydrolysis, which is followed by peptide release (Flynn et al., 1989). Kar2p may be an acceptor for nascent translocating proteins, keeping the emerging peptide in an unfolded conformation until translocation is complete. This would prevent aberrant or premature folding from occurring, especially that mediated by hydrophobic interactions (for reviews see Pelham, 1986; Rothman, 1989; Gething and Sambrook, 1992). Alternatively, by analogy to the requirement for dnaK and dnaJ during λ replication (Zylicz et al., 1989), Kar2p and Sec63p might interact during translocation to activate the translocation apparatus.

Other hsc70 molecules are also involved in protein translo-

1. *Abbreviations used in this paper:* hsc, heat-shock protein; pαF, proα-factor; ppαF, prepro-α-factor; SRP, signal recognition particle.

cation. In *S. cerevisiae*, the hsc70s are encoded by at least eight genes, including a subfamily known as the *SSA* genes (Werner-Washburne et al., 1987). When cells are depleted of the *SSA1* gene product in a strain that has the *SSA2* and *SSA4* genes deleted, untranslocated ER and mitochondrial precursor proteins accumulate (Deshaies et al., 1988). PpαF translated in a yeast extract can be posttranslationally translocated into yeast microsomes (Hansen et al., 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986). The inefficient translocation of wheat germ–translated ppαF can be corrected by the addition of Ssalp or Ssa2p (Chirico et al., 1988; Deshaies et al., 1988). Cytosolic hsc70's may interact with preproteins, keeping them in a translocation-competent state until they are translocated. In mammalian cells, signal recognition particle (SRP) may play this role (Crooke et al., 1988).

A further definition of this process requires that purified and functional membrane components be reconstituted into lipid vesicles. When the *E. coli* proteins SecY/E are reconstituted into liposomes in the presence of SecA and a source of ATP and membrane potential, the *E. coli* preprotein proOmpA is translocated into the vesicles (Brundage et al., 1990; Driessen and Wickner, 1990). Reconstitution of translocation into the mammalian ER from a detergent-solubilized ER fraction has been achieved (Nicchitta and Blobel, 1990), and the initial identification of fractions and proteins required for translocation has begun (Nicchitta and Blobel, 1991; Simon and Blobel, 1992; Görlich et al., 1992).

In yeast, one may rely on genetics to identify the reconstitution of proteins that are essential for translocation. We now present a method for solubilizing yeast microsomes in detergent, and reconstituting protein translocation into liposomes after removal of the detergent. The method faithfully reproduces aspects of the translocation reaction in microsomes, such as signal peptide cleavage, dependence on ATP, and the presence of wild-type gene products known to be essential for translocation in vivo. We also demonstrate that Kar2p and Ssalp play unique roles during translocation.

## Materials and Methods

### General Materials

Yeast strains used were RSY607 (*MATα, ura3-52, leu2-3, -112, pep4::URA3*, N. Pryer, this laboratory), RSY151 (*MATα, sec63-1, ura3-52, leu2-3, -112, pep4-3*; Rothblatt et al., 1989). RSY156 (*MATα, ura3-52, leu2-3, -112, pep4-3*; Rothblatt et al., 1989), MW141 (*MATα, ura3-52, leu2-3, -112, trp1, his3-11, -15, ssa1::HIS3, ssa2::LEU2, ssa4::URA3, pGAL1-SSA1-TRP1*; Deshaies et al., 1988), and MS137 (*MATα, kar2-159, ura3-52, leu2-3, -112, ade2-101*, Vogel et al., 1989). Antibodies to SEC61p (Stirling et al., 1992), and Sec62p (Deshaies and Schekman, 1990) have been described, and antibodies to Sec63p were generated against a protein A-Sec63 fusion protein (Feldheim et al., 1992). Antibodies to Kar2p have been described (Rose et al., 1989) and were a gift from the Rose Laboratory (Princeton University, Princeton, NJ). The dnaK protein was generously provided by the H. Echols laboratory (University of California, Berkeley, CA).

### Preparation of Yeast Microsomes and Reconstituted Proteoliposomes

Yeast cells were grown in YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose), to a final OD at 600 nm of 2–4 at either 20°C for the *sec* or *kar* mutant strains or 30°C for wild-type strains (see above). Yeast microsomes were prepared essentially as described (Rothblatt and Meyer, 1986), with minor modifications (Deshaies and Schekman, 1989). The final membrane pellet after homogenization was washed once, then resuspended in buffer 88 (20 mM Hepes, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc) to a final concentration of ~10 mg protein/ml ($OD_{280}$ = 40 in 1% SDS), and finally quick-frozen in liquid nitrogen in 0.05-ml aliquots and stored at −70°C. Azolectin containing 45% phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) was dissolved by vigorous stirring in lipid buffer (10 mM $KP_i$, pH 7.0, 2 mM β-mercaptoethanol) at a concentration of 50 mg/ml and frozen under nitrogen at −70°C. Liposomes were prepared from thawed 1-ml aliquots of azolectin using the microtip on a Heat Systems Sonicator (Heat Systems Inc., Farmingdale, NY) at 40 W output until the solution cleared (~1 min). The solution (1 ml) was cleared by centrifugation at 100,000 *g* (model TLA 100.3; Beckman Instruments, Inc., Palo Alto, CA) for 30 min which removed multilamellar lipid sheets from the supernatant as determined by EM. The clear supernatant was stored at 4°C for 24 h until needed. Ultrol grade octyl-β-D-glucopyranoside (Calbiochem Corp., La Jolla, CA) was prepared in sterile water at a concentration of 10% and stored at −20°C.

To prepare the detergent extract for reconstitution, 50 μl of microsomes (10 mg protein/ml) were dispersed in 0.36 ml of solubilization buffer (100 mM $KP_i$, pH 7.0, 500 mM KOAc, 10 mM DTT, 20% glycerol) followed by the addition and mixing of 30 μl of the cleared liposomes. Under these conditions, the final exogenous lipid to protein ratio was 3:1. Octylglucoside (60 μl of 10%) was then layered on the mixture, rapidly agitated at the highest setting of a Vortex mixer for 3–5 s (final concentration of detergent was 1.2%), and the cleared solution was left on ice for 15–30 min. The extract was centrifuged at 100,000 *g* in a Beckman TLA 100.3 rotor for 30 min at 4°C (300,000 *g* could also be used with no change in reconstituted translocation efficiency, implying that residual membrane contamination was not responsible for the observed reconstitution activity). The supernatant solution was placed in 1-cm Spectraporl dialysis tubing (6,000 mol wt cut-off; Spectrum Medical Industries, Inc., Los Angeles, CA) and dialyzed against 1,000 vol of 20 mM $KP_i$, pH 7.0, 150 mM KOAc, 2 mM β-mercaptoethanol, and 20% glycerol for 15–17 h at 4°C. An aliquot (150 μl) of the turbid dialysate was gently mixed with 0.30 ml of the cleared liposomes in a glass culture tube (lipid to protein ratio of approximately 100:1). A dry ice–acetone bath was prepared into which the tube was immersed for 10–20 s. After thawing at room temperature, the reconstituted vesicles were concentrated twofold by centrifugation in an Amicon Centricon 30 Microconcentrator (Amicon, Beverly, MA) at 3,000 *g* in a SS-34 rotor for 45 min. The resultant vesicles were competent for translocation for up to 4 h if left on ice, and the detergent extract before dialysis could be stored at −20°C in 50% glycerol without loss of activity for at least a week.

### Translocation Assay

Reactions typically contained 60 μl, which included an ATP regenerating system (1 mM ATP, 50 μM GDP-mannose, 40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase) or 1 mM ATPγS (indicated by "−ATP" in the figures and text; the absence of nucleotide or addition of ATPγS reduced translocation to the same level), 2 μl of microsomes or 20 μl of reconstituted vesicles prepared as described above, and buffer 88. As indicated, 300 μg protein of a 300,000 *g* supernatant fraction from a yeast cytosol fraction prepared using the nitrogen lysis procedure (Sorger and Pelham, 1987) was included in the assays performed with the reconstituted vesicles. Addition of cytosol enhanced the level of translocation by zero to fourfold, depending on the preparation of radiolabeled ppαF. Cytosol alone did not support the appearance of protease-protected pαF. Reactions were commenced by the addition of ~200,000 cpm of either yeast (Rothblatt and Meyer, 1986) or wheat germ (Steuber et al., 1984) in vitro–translated $^{35}$S-ppαF. Reactions were conducted for 40 min at 20°C. The reaction plateaued at 1 h, and was inhibited at 4°C (data not shown). Aliquots (20 μl) were removed and either mixed with TCA to a final concentration of 20%, or mixed with trypsin (final concentration 0.25 mg/ml), or trypsin and Triton X-100 (final concentration 1%), and incubated at 0°C for 45 min. Proteolysis was quenched by the addition of TCA, and the reactions were left on ice for another 15 min, after which they were centrifuged in a refrigerated Tomy microcentrifuge for 10 min. The pellet fractions were washed once with ice-cold acetone and recentrifuged. The final pellet fractions were resuspended in 25 μl of Laemmli sample buffer, heated to 95°C for 3 min, and the samples were resolved on a 10% polyacrylamide gel containing SDS (Laemmli et al., 1970). The gels were fixed in 10% HOAc containing 25% isopropyl alcohol, dried, and exposed to a Phosphorimaging screen for 1.5 d. Results were quantified and visualized using a PhosphorImager from Molecular Dynamics (Sunnyvale, CA). Background radiation on each gel was subtracted from the values presented in Figs. 4 and 6.

### Sucrose Gradient Analysis of Reconstituted Vesicles

Reconstituted proteoliposomes were prepared and either directly mixed in 1 ml of sucrose to a final concentration of 55% (wt/vol), or were first carried through a translocation assay and trypsin treatment before being mixed with sucrose. Sucrose solutions of 0, 15, 25, 35, and 45% (wt/vol) in lipid buffer (see above) were prepared, and 0.8 ml of each were successively layered onto the 55% sucrose solution containing the vesicles in a 5 ml ultracentrifuge tube (13 × 51 mm) (Beckman Instruments, Inc.). The gradients were centrifuged at 100,000 *g* for 24–30 h at 4°C, and fractionated using an ISCO Gradient Fractionator (ISCO, Lincoln, NE). The 0.3-ml fractions were either subjected to scintillation counting and SDS-PAGE followed by autoradiography (see above), or SDS-PAGE for silver staining and immunoblotting, depending on whether a translocation reaction was performed. Sucrose percentages (wt/wt) were determined using a Zeiss refractometer (Carl Zeiss, Inc., Thornwood, NY).

### Electron Microscopy

Samples for EM were fixed in 2% glutaraldehyde, 1% paraformaldehyde, and 0.1 M sodium cacodylate for 2 h at 4°C, washed once in buffer, then postfixed for 1 h at 4°C in 1% osmium tetroxide and 1.5% potassium ferricyanide. After three buffer washes, the samples were transferred in turn to the following solutions: 0.25% tannic acid in 50 mM cacodylate (30 min), buffer (wash), 0.5% sodium sulfate (5 min), distilled water (three washes), 2% uranyl acetate (1 h), distilled water (three washes), and finally dehydrated in an ethanol series. Several absolute ethanol changes were made before overnight infiltration with a 1:1 mixture of ethanol:Spurr's resin. After two changes of 100% resin over 2–4 h, the samples were embedded in fresh resin and polymerized at 60°C for 24 h. Thin sections were cut and stained with 2% uranyl acetate and lead citrate, and examined on an electron microscope (model 301; Philips Electronic Instruments Co., Mahwah, NJ).

### Purification of Ssalp and Kar2p

One liter of MW141 yeast cells (Deshaies et al., 1988) were grown at 30°C to a final $OD_{600}$ of 2 in YPG (1% Bacto-yeast extract, 2% Bacto-peptone, 2% galactose) to induce overexpression of the *SSA1* gene from the *GAL1* promoter. All subsequent steps were performed at 4°C. Cells were collected by centrifugation at 4,000 *g* for 5 min, washed once with water, and processed as described (Chappell et al., 1986). Half of the 100,000 *g* supernatant fraction was applied to a 5-ml ATP-agarose column (Sigma Chemical Co., St. Louis, MO) (C8 linkage, 9 atom spacer) eluted at 0.4 ml/min, and washed successively with 5 vol of buffer C (20 mM Hepes, NaOH, pH 7.0, 25 mM KCl, 2 mM MgOAc, 0.8 mM DTT), buffer C plus 1 M KCl, and finally buffer C again. ATP-binding proteins were eluted with buffer C containing 1 mM ATP. The 1-ml fractions which harbored Ssalp (as readily seen by a prominent band migrating at 70 kD on SDS-PAGE) were pooled and applied to a 4 ml DEAE-Sepharose column equilibrated in buffer C. After washing the column with 5 vol of buffer C, proteins were eluted by a 50-ml linear salt gradient from 25–700 mM KCl in buffer C. Ssalp was >95% pure in the fraction eluting at 200 mM salt. The yield from 1,000 $OD_{600}$ of cells was 1.1 mg of Ssalp as assessed using the method of Lowry et al. (1951) and by comparing protein to BSA standards electrophoresed in parallel and silver stained.

To purify Kar2p, one liter of a *pep4*-deleted strain (RSY607) was grown to an OD of 4 at 30°C, and the cells were washed and lysed as for the Ssalp purification. The broken cells were centrifuged at 20,000 *g* for 10 min and the pellet was washed once in buffer 88. The pellet was resuspended in 50 ml of buffer 88 containing 0.2% saponin and mechanically rotated at 4°C for 30 min. After centrifugation again at 20,000 *g* for 10 min, 200 mg of total protein in the supernatant was applied to a 10-ml Pharmacia Q-Sepharose FPLC column (Pharmacia Fine Chemicals, Piscataway, NJ). After washing with 20 ml of buffer 88, the salt concentration was increased to 0.75 M KOAc, and the column was washed with another 20 ml. A 40-ml linear gradient to 2 M KOAc was applied, 1-ml fractions were collected, and Kar2p was detected by immunoblotting with an anti-Kar2p antibody (Rose et al., 1989). Kar2p eluted from Q-Sepharose at ~0.80 M KoAc. The pooled Kar2P fractions were loaded onto an ATP-agarose column equilibrated and eluted as for the Ssalp purification. The ATP eluate was subsequently loaded onto a 5-ml DEAE-Sepharose column equilibrated in buffer C and eluted at 0.3 ml/min. After washing with 25 ml of 200 mM KCl (which removed any Ssalp contamination that was present), Kar2p was eluted with 1 column vol of buffer C plus 300 mM KCl. The Kar2p was >95% pure with a final yield of 50–100 μg of protein based on a comparison of silver-stained protein and BSA electrophoresed in parallel. The final fraction contained ATP which interferes with the Lowry assay (Lowry et al., 1951). Removal of ATP by subsequent steps significantly decreased the yield, and there was a loss of Kar2p activity in a *kar2-159* reconstitution mixing assay.

## Results

### Reconstitution and Analysis of the Proteoliposomes

Reconstitution of translocation with detergent-solubilized membranes relies on the assay procedure developed with intact yeast membranes. When yeast microsomes are incubated with an ATP regenerating system in the presence of $^{35}$S-ppαF prepared from a yeast in vitro translation reaction, ppαF is translocated into microsomes. The signal peptide is then cleaved and the pro-α-factor (pαF) becomes triply glycosylated (3gpαF), changing its apparent molecular weight from 18,000 to 28,000 (Hansen et al., 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986). An example of this reaction is depicted in Fig. 1 *B*, lane *1*. On addition of trypsin, the untranslocated ppαF is degraded, while a portion of the 3gpαF is protected (Fig. 1 *B*, lane *2*). The 3gpαF is protected by the integrity of the membrane because it too is degraded by trypsin when detergent is included during proteolysis (Fig. 1 *B*, lane *3*). Given the fact that glycosylation is not essential for translocation, we wished to establish electrophoretic mobility standards that would detect signal peptide-processed but unglycosylated pαF. To visualize the conversion of ppαF to pαF by yeast signal peptidase (Böhni et al., 1988), cells were grown to log phase and treated for 1 h with 10 μg/mL of the glycosylation inhibitor, tunicamycin. When yeast microsomes were prepared from these cells and tested for translocation activity, only protected pαF, but not 3gpαF was visible (Fig. 1 *A*, lane *2*). In this gel system, pαF migrates more slowly than ppαF, even though it is shorter by 19 amino acids (Waters et al., 1988).

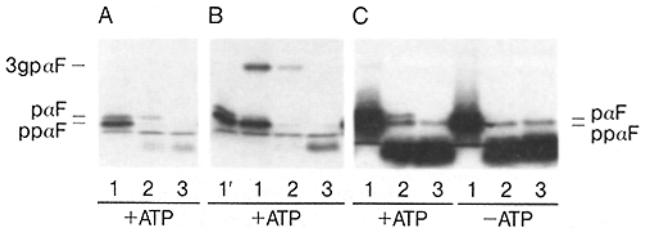


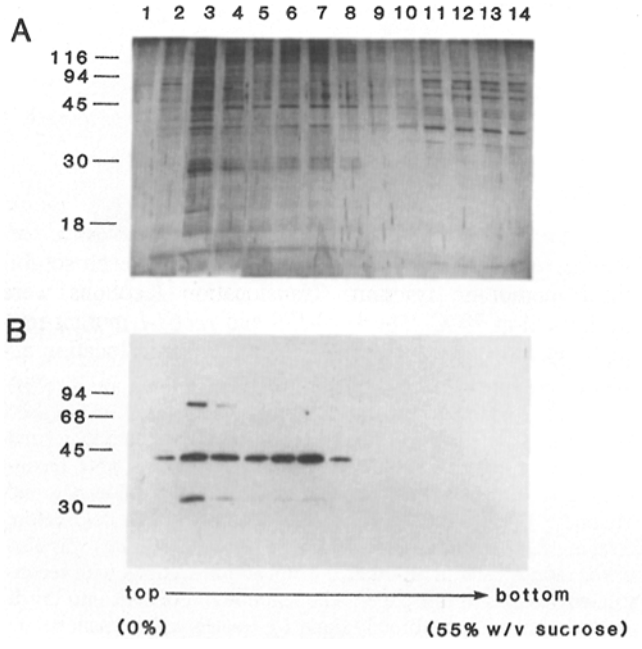

*Figure 1.* Comparison of ppαF translocation into untreated, tunicamycin-treated, and reconstituted microsomes. Yeast microsomes were prepared from cells grown either in the presence (*A*) or absence (*B* and *C*) of tunicamycin, as described in the text. Microsomes were assayed directly for translocation activity (*A* and *B*), or were detergent solubilized, and the proteins were reconstituted into liposomes (*C*), as outlined in the Materials and Methods. The proteoliposomes were concentrated twofold before assaying for translocation of $^{35}$S-ppαF. Cytosol (300 μg) was also included in *C*, and in all other translocation reactions with reconstituted vesicles in this paper. The reactions were split into thirds and TCA precipitated directly (lane *1*), treated with trypsin before precipitation (lane *2*), or trypsin treated in the presence of detergent before precipitation (lane *3*). Lanes *A1* and *B1'* represent identical samples. *–ATP* series corresponds to the addition of ATPγS (see Materials and Methods).

In our attempt to reconstitute protein translocation from detergent-solubilized microsomes, we followed the protocols using *E. coli* or dog pancreas membranes as the starting material (Brundage et al., 1990; Driessen and Wickner, 1990; Nicchitta and Blobel, 1990). The first successful experiments, however, followed the procedure used to reconstitute the yeast glucose transporter (Franzusoff and Cirillo, 1982). In this protocol, membrane proteins were solubilized with octylglucoside in a buffer that contained azolectin (soybean) phospholipid liposomes and glycerol (see Materials and Methods). After centrifugation to remove insoluble material, the supernatant was dialyzed to remove detergent, and then mixed with a large excess of liposomes. Upon freeze thawing the mixture, proteoliposomes were formed. In our procedure, the reconstituted vesicles were concentrated twofold and yeast cytosol was added to the translocation reactions (see Materials and Methods). The assumption was that some loosely associated membrane proteins that are essential for translocation in microsomes were lost during the reconstitution.

Reconstituted vesicles mixed with $^{35}$S-pp$\alpha$F generated trypsin-protected p$\alpha$F in the presence but not in the absence of ATP (Fig. 1 *C*, see lane 2 *+ATP* versus *−ATP*, upper band). This material was degraded by the addition of trypsin and 1% Triton X-100 to the reaction (Fig. 1 *C*, lane *3 +ATP*), suggesting that the p$\alpha$F was membrane protected. The species that migrated at the position of 3gp$\alpha$F in the reaction with the reconstituted vesicles (Fig. 1 *C*, lane *I*) probably was aggregated pp$\alpha$F, because higher molecular weight oligomers of pp$\alpha$F were also evident on this gel (not shown). Quantitation by PhosphorImager analysis showed that the formation of protease protected p$\alpha$F was stimulated 3.5-fold by ATP, and the overall efficiency of the reaction (the amount of ATP-dependent protease-protected p$\alpha$F divided by the total amount of label in the reaction) was ~10%. By comparison, the efficiency reported for reconstitution of the mammalian translocation apparatus was 30–40% (Nicchitta and Blobel, 1990). This discrepancy arises since yeast microsomes are only somewhat enriched in ER. Furthermore, the starting translocation efficiency is only ~50% (see Figs. 1 *B* and 7 *B*). Reconstitutions with a wide variety of other detergents or lipids failed to increase this efficiency.

Unlike the formation of sequestered p$\alpha$F, the amount of protease-protected pp$\alpha$F was not distinguishable in translocation reactions conducted in the presence or absence of ATP. Increasing amounts of trypsin in the presence or absence of Triton X-100 failed to degrade this material (Fig. 1 *C*, lane *3*, *+ATP* and *−ATP*). Protease insensitive pp$\alpha$F was detected with proteoliposomes or pure phospholipid vesicles, and was not reduced by urea denaturation of the precursor, but was not observed when no membranes or native membranes were used in a translocation reaction. This signal probably represents aggregation of pp$\alpha$F on the surface of the phospholipid vesicles. Given this anomalous behavior of a fraction of the pp$\alpha$F, the formation of protease-protected p$\alpha$F was selected as a more reliable measure of translocation activity.

Reconstituted vesicles were characterized by density gradient centrifugation. A 0–55% (wt/vol) sucrose gradient was prepared in which the vesicles occupied the bottom layer, and the samples were centrifuged until equilibrium was established. Under these conditions, the phospholipid vesicles should "float-up" to achieve equilibrium density whereas protein aggregates or proteins not associated with the liposomes should remain in the bottom layer. A protein profile of this gradient is presented in Fig. 2 *A*. When these samples were immunoblotted, Sec61p, Sec62p, and Sec63p comigrated at 13% wt/wt sucrose (Fig. 2 *B*, fraction 3): Sec61p,



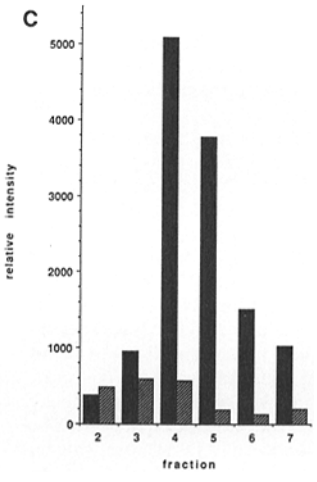




*Figure 2.* Enrichment of reconstituted vesicles by density centrifugation. Reconstituted proteoliposomes were mixed with sucrose to a final concentration of 55% (wt/vol), and centrifuged to equilibrium on a 0–55% (wt/vol) sucrose gradient as described in Materials and Methods. The gradient was fractionated and aliquots were subjected to SDS-PAGE and either silver stained (*A*) or transferred to nitrocellulose and immunoblotted for the presence of Sec63p (apparent mol wt = 71,000), Sec61p (apparent mol wt = 42,000), and Sec62p (apparent mol wt = 32,000) using the ECL system (Amersham Corp., Arlington Heights, IL) (*B*). Fraction 3 contained 13% (wt/vol) sucrose. (*C*) The reconstituted vesicles were added to a translocation reaction either in the presence (■) or absence (▨) of ATP, as in Fig. 1 *C*. The reactions were treated with trypsin and centrifuged on a sucrose gradient as in *A* and *B*. After centrifugation, the gradient was fractionated, and the proteins were TCA precipitated and resolved by SDS-PAGE. The amount of p$\alpha$F in each gradient was quantified on a PhosphorImager. The y-axis represents relative densitometry units.

apparent mol wt = 42,000; Sec62p, apparent mol wt = 32,000; and Sec63p, apparent mol wt = 71,000. Kar2p also peaked in this fraction, as detected by immunoblotting, and coincided with a band of white lipid. Quantitative immunoblotting showed that between 10 and 25% of the total Kar2p from microsomes floated to this fraction. Vesicle-enclosed Kar2p was insensitive to trypsin, but was completely degraded by trypsin in the presence of 1% Triton X-100 (not shown).

Sec61p was present in many fractions that floated in the gradient (Fig. 2 *B*, lanes *2–8*). Deshaies et al. (1991) reported that Sec61p is more abundant than Sec62p and Sec63p, and that only a fraction of the Sec61p is associated with the Sec protein complex. It is possible that Sec61p dissociated from the other Sec proteins in the complex during the flotation, or that multiple populations of vesicles exist, one of which contains Sec61p without the other proteins. Density centrifugation also resolved a significant amount of other protein that did not comigrate with the Sec proteins in the vesicles (see Fig. 2 *A*, lanes *10–14*). This procedure is, therefore, a partial purification for proteins presumed to be involved in translocation.

Because the proteoliposomes at 13% wt/wt sucrose contained the Sec proteins, we tested whether these membranes contained the protease-protected pαF. Reconstituted vesicles were incubated in a translocation reaction with or without ATP and membranes were exposed to trypsin to remove any untranslocated, accessible ppαF, and brought up in a sucrose solution to a final concentration of 55% (wt/vol). A sucrose density gradient was centrifuged and fractions were monitored by SDS-PAGE. Membranes containing ATP-dependent pαF equilibrated at 12.5–15.5% (wt/wt) sucrose (fractions 4 and 5 in Fig. 2 *C*), approximately the same density region in which membranes containing the Sec proteins equilibrated. Attempts to translocate ppαF into the vesicles after enrichment by sucrose gradient fractionation were unsuccessful.

Reconstituted proteoliposomes were isolated from a sucrose gradient and compared with native yeast microsomes by thin section EM. As shown in Fig. 3 *A*, yeast microsomes are an extremely heterogeneous mixture of membranes, vesicular structures, and electron-dense material. The reconstituted proteoliposomes by comparison (Fig. 3 *B*) appear to be 50–200-nm vesicles, containing a mixture of uni- and multi-lamellar structures.

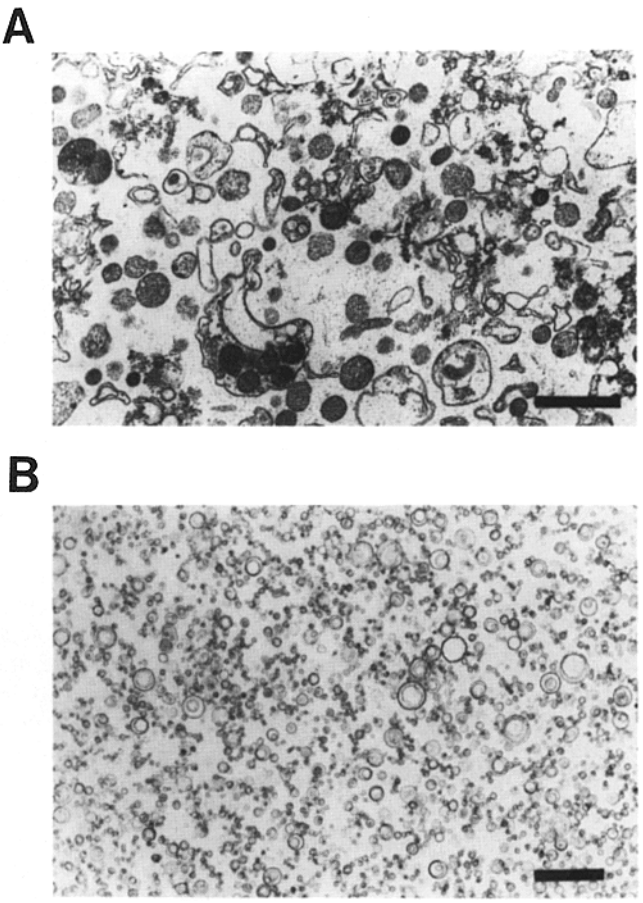



*Figure 3.* Electron microscopy of yeast microsomes (*A*) and sucrose gradient-enriched proteoliposomes (*B*). Microsomes and reconstituted vesicles were prepared and fixed while in solution for visualization by EM (see Materials and Methods). Bars: (*A*) 1 μm; (*B*) 0.5 μm.

### *Dependence of Translocation on Genetically Identified and Purified Factors*

Mutations in several genes affect translocation of preproteins into the ER. At the nonpermissive temperature, *sec63-1* mutant cells accumulate the untranslocated precursors of ppαF, the periplasmic enzyme acid phosphatase, and the vacuolar enzyme carboxypeptidase Y (Rothblatt et al., 1989). The temperature-sensitive defect in ppαF translocation can be reproduced in vitro using microsomes incubated at 33°C but derived from mutant cells grown at the permissive temperature of 20°C (Rothblatt et al., 1989). Similarly, the *kar2-159* mutation in cells grown at the nonpermissive temperature blocks the translocation of ppαF, secreted invertase, carboxypeptidase Y, and the *KAR2* gene product (BiP) itself (Vogel et al., 1990). Microsomes prepared from mutant *kar2-159* strains grown at the permissive temperature are defective in an in vitro assay for ppαF translocation at any temperature (Sanders et al., 1992).

We examined reconstituted vesicles prepared from membranes from mutant cells. Mutant and the respective isogenic wild-type strains were grown at 20°C, microsomes were prepared, and reconstitution was performed with each solubilized membrane fraction. Translocation reactions were performed at 20°C. The *kar2-159* and *sec63-1* mutant proteoliposomes were ~threefold reduced in translocation activity, as measured by the relative amounts of trypsin-protected pαF (Fig. 4). The activity exhibited by the mutant proteoliposomes was similar to that of wild-type proteoliposomes incubated in the absence of ATP. We conclude that translocation into reconstituted vesicles is dependent on Kar2p, and that *sec63-1* reconstituted vesicles do not support translocation.

The *sec63-1* mutant showed a severe translocation defect in reconstituted vesicles when assayed at the permissive temperature of 20°C (Fig. 4). When *sec63-1* microsomes were assayed at the permissive temperature, however, the translocation defect was less pronounced (Rothblatt et al., 1989). In contrast, the *kar2-159* mutation in microsomes displayed a severe translocation defect at any temperature (Sanders et

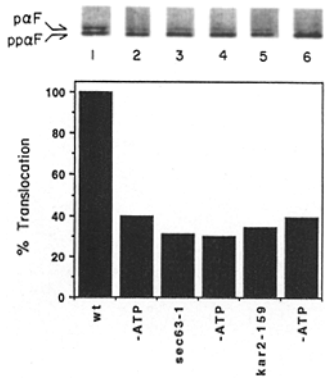



*Figure 4.* Reconstituted vesicles prepared from *sec63-1* and *kar2-159* microsomes are defective for translocation. Microsomes and the corresponding proteoliposomes were prepared as described in the Materials and Methods. The reconstituted vesicles were assayed for translocation as in Fig. 1 *C*, either in the presence or absence of ATP (where indicated), and the samples were trypsin treated to remove any accessible substrate. (*Top*) Digital images showing protease-resistant ppαF and pαF; (*bottom*) quantitation of the amount of protease-protected pαF from the upper image. Wild-type level (absolute translocation and processing efficiency of ~10%; see text for details) is taken to represent 100% translocation.

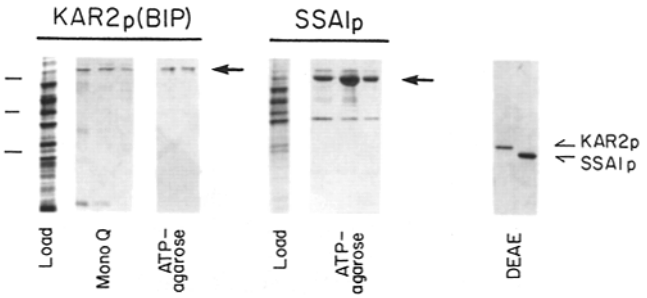



*Figure 5.* Purification of Kar2p and Ssalp from *S. cerevisiae*. Proteins were purified as described in Materials and Methods. Shown here are only the fractions from each purification step that were pooled for use in the next purification step. Markers on the far left (top to bottom) correspond to molecular weight standards of 68,000, 45,000, and 30,000, respectively. Proteins were visualized by silver staining 12.5% SDS-polyacrylamide gels: far right, final purified proteins after DEAE-Sepharose, as distinguished on a 10% SDS-polyacrylamide gel.

al., 1992), and was also completely restrictive in the reconstituted vesicles (Fig. 4). The reason for this discrepancy is unknown. Possibly, the *sec63-1* mutation does not allow a translocation complex to reassemble properly from the detergent extract, while complex assembly is disturbed in microsomes only at higher temperatures. The *kar2-159* mutation may affect the integrity of the complex in either microsomes or reconstituted vesicles.

Next we evaluated mutant vesicles reconstituted in the presence of a pure wild-type protein. To perform this experiment, Kar2p, as well as the yeast cytoplasmic hsc70 homologue, Ssalp (Werner-Washburne et al., 1987), were purified to homogeneity. Fig. 5 shows the peak fractions after each purification step for Kar2p and Ssalp. The purified proteins were included during a dialysis with the solubilized membrane fractions to allow incorporation into forming vesicles. Reconstituted vesicles were assayed and as before wild-type vesicles were active (Fig. 6, lane *1*), while *kar2-159* vesicles (lane *5*) were not. However, with increasing amounts of purified Kar2p included during reconstitution of a Kar2 fraction, a translocation efficiency of ~90% of the wild type was achieved (Fig. 6, lanes *2–4*). The pαF signal in these samples was sensitive to trypsin in the presence of Triton X-100 (data not shown). The amount of purified Kar2p that restored this activity represented 1% of the total protein in the detergent extract. This amount of protein is not unreasonable given that BiP has been estimated to represent ~1% of the total protein in the mammalian ER, or ~5% of the lumenal content of the ER (for reviews, see Rothman, 1989; Gething and Sambrook, 1992). Including BSA, Ssalp, or the *E. coli* hsp70 homologue dnaK during dialysis failed to replace the requirement for BiP (Fig. 6, lanes *6–8*, respectively). We found that Kar2p, added after the vesicles were formed or when the dialysate and liposomes were frozen and thawed, failed to rescue the defect. ATP (2 mM) alone also failed to rescue the translocation defect when present during the dialysis of the *kar2-159* membrane extract. When Ssalp was reconstituted with solubilized *kar2-159* microsomes, and the vesicles were treated with trypsin and floated in a sucrose gradient as described before, Ssalp appeared to efficiently associate with the vesicles. Together, these data support the view that BiP participates directly in the translocation reaction in a manner that is not satisfied by other hsc70 isozymes.

Cytosolic hsc70 (Ssalp in yeast) stimulates the translocation of ppαF in vivo and in a heterologous translation/translocation reaction comprising a wheat germ-soluble fraction and yeast microsomes (Chirico et al., 1988; Deshaies et al., 1988). We reproduced the in vitro result with pure Ssalp (Fig. 7). By contrast Kar2p and BSA failed to restore this activity. dnaK, however, marginally restored activity. This result demonstrates that pure Ssalp performs some essential role in the preparation of ppαF for translocation that is not satisfied by other hsc70 isozymes such as Kar2p. Pure Ssalp failed to replace the cytosolic requirement for translocation using wheat germ-translated ppαF and the reconstituted vesicles (not shown). It is likely that a necessary component(s) in addition to Ssalp was lost during the reconstitution.

## Discussion

We have devised a procedure that allows solubilized yeast membranes to be reconstituted into proteoliposomes that are competent for the translocation and signal peptide processing of yeast α-factor precursor. The reconstituted vesicles reproduce the physiological requirements for ATP and the Sec63 and Kar2 (BiP) proteins. Proteoliposomes prepared

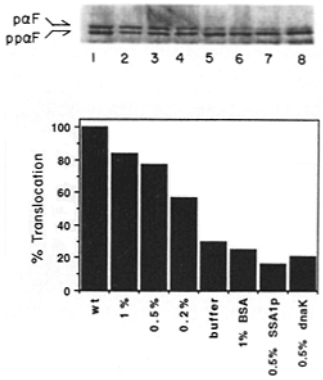



*Figure 6.* Purified Kar2p (BiP) corrects the *kar2-159* defect in reconstituted vesicles. Reconstituted proteoliposomes from either a *kar2-159* strain or the isogenic wild-type strain were prepared and assayed for translocation activity as in Fig. 5. (*Top*) Digital image showing protease-protected ppαF and pαF (*bottom*) quantitation of the relative amounts of protected pαF. Wild-type level of translocation (absolute translocation and processing efficiency ~10%) is taken to be 100%.

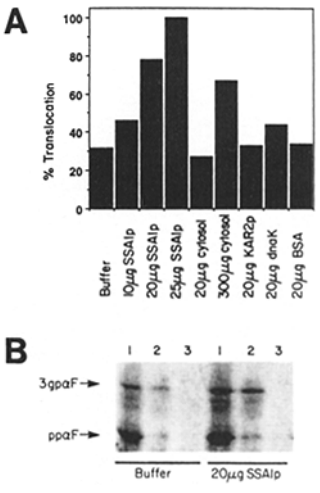



*Figure 7.* Ssalp rescues the wheat germ-translated ppαF defect in microsomes. Wild-type microsomes and wheat germ–translated ppαF were prepared as described in the Materials and Methods. Translocation reactions were performed and assayed as in Fig. 1 *B*. (*A*) Percent translocation apparent when the indicated proteins were added to the reactions. Complete (100%) translocation is defined as the amount of protected 3gpαF signal present when 25 μg of Ssalp was added to a reaction with wheat germ–translated ppαF and microsomes. (*B*) Original images from reactions with either no addition (*Buffer*), or 20 μg of Ssalp. Lanes represent total reactions (lane *1*); trypsin-treated samples (lane *2*); or trypsin-treatment in the presence of Triton X-100 (lane *3*).

from *kar2-159* cells are unable to translocate ppαF, but the defect is corrected by the addition of pure Kar2p before proteoliposome formation. Cytosolic hsc70 (yeast Ssalp) is unable to replace Kar2p in reconstituted vesicles. As previously shown (Chirico et al., 1988; Deshaies et al., 1988), Ssalp acts as a chaperone to promote the translocation of ppαF into native yeast microsomes. Kar2p does not substitute for Ssalp in this reaction.

Proteins of the hsc70 family bind and release polypeptides concomitant with ATP hydrolysis. This property may explain how hsc70 isozymes function as "unfoldases" or "molecular chaperones" to prevent protein aggregation, facilitate folding and translocation, or stabilize interactions between proteins (reviewed by Pelham, 1986; Ellis, 1987; Rothman, 1989; Gething and Sambrook, 1992). The *KAR2* and *SSA1* genes predict proteins of 63% identity (Rose et al., 1989). In the simplest view, the only functional difference between Kar2p and Ssalp is that one is transported into and resides in the ER by virtue of its signal and ER retention peptides (Normington et al., 1989; Rose et al., 1989), while the other is cytosolic. Accordingly, Ssalp keeps nascent secretory proteins from folding in the cytosol until they are translocated into the ER, while Kar2p receives translocating polypeptides in the ER and prevents aberrant folding until translocation is complete. If Ssalp and BiP have equivalent activities, they should be functionally interchangeable. Our data argues against this simple view: Ssalp and Kar2p did not replace each other in translocation reactions designed to measure the participation of each.

There are several explanations for the lack of Ssalp and Kar2p exchangeability. The two proteins may possess the same activity but function at radically different concentrations. In the range of protein concentrations used in our experiments, Kar2p and Ssalp may exhibit important differences in affinities for their substrates. Unidirectional translocation might be facilitated by a higher affinity or concentration of Kar2p in the lumen of the ER in respect to the affinity or concentration of Ssalp in the cytosol.

It seems unlikely that distinct affinities of Kar2p and Ssalp for α-factor precursor would be the sole determinant of topological specificity. The presence of a signal peptide on ppαF may promote the recruitment of specificity factors that ensure selective association of Ssalp with other domains of the precursor. Conversely, in the ER lumen, BiP may contact a translocating chain only after signal peptide cleavage, and may do so only in the context of precursor association with a channel protein such as Sec61p (Sanders et al., 1992). In the absence of the proper determinants of specific interaction, the two hsc70 isozymes, though possessing the same intrinsic peptide-binding activity, may fail to replace each other.

Kar2p specificity may develop from interactions that are not directly linked to a translocating polypeptide. Genetic interaction between *kar2* and *sec63* alleles has been detected (M. Scidmore and M. Rose, personal communication). A rational basis for physical interaction between Kar2p and Sec63p develops from the discovery of an ER lumenally oriented domain of Sec63p that has significant homology to the $NH_2$ terminus of the *E. coli* dnaJ protein (Sadler et al., 1989; Feldheim et al., 1992). In *E. coli* the dnaJ and hsc70-like dnaK proteins interact to fulfill roles in λ phage DNA replication (Yamamoto et al., 1987). Hence, it seems likely that Kar2p may be recruited to the lumenal surface of the translocation complex through an association with Sec63p. Conversely, a distinct cytosolic dnaJ homolog in yeast such as Ydjlp or Sislp (Caplan and Douglas, 1991; Luke et al., 1991) could serve to position cytosolic hsc70 on the cytosolic face of the ER.

The participation of hsc70 isozymes in polypeptide translocation into the mammalian ER has not yet been documented. Cotranslational translocation of mammalian secretory proteins may require SRP and not cytosolic hsc70, whereas posttranslational translocation of ppαF into yeast ER requires both SRP and cytosolic hsc70 (Stirling and Hewitt, 1992; Deshaies et al., 1988). Kar2p appears to be required both for post-translationally translocated molecules such as ppαF and for cotranslationally translocated molecules such as invertase (Rothblatt and Meyer, 1986; Vogel et al., 1990). Nevertheless, translocation reactions reconstituted from permeabilized or solubilized dog pancreas ER fractions are depleted of BiP with no apparent drop in translocation activity (Bulleid and Freedman, 1988; Nicchitta and Blobel, 1990). It is possible that the difference in dependence of the mammalian and yeast reactions on BiP/Kar2p may point to mechanistic differences in the two processes. However, it is equally plausible that BiP functions catalytically in protein translocation, and amounts undetectable by immunological means may be sufficient to support the translocation of minute radiochemical concentrations of secretory polypeptides.

In addition to illuminating the distinct roles of cytosolic and lumenal hsc70 isozymes, the reconstitution method described here should allow the complete resolution of the integral membrane proteins required for translocation. Such has already been achieved with *E. coli* inner membrane proteins (SecY and SecE; Brundage et al., 1990), and good progress is being made with a reconstituted protein translocation reaction from dog pancreas ER (Nicchitta et al., 1991; Görlich et al., 1992). Preliminary results demonstrate that a highly purified fraction of Sec63p restores translocation activity to proteoliposomes reconstituted from *sec63* mutant mem-

branes. Similar biochemical complementation is anticipated as a means to assay and purify functional forms of Sec61p and Sec62p.

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