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

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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.

Translocating ppeF 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 (Zylic and others, 1989), Kar2p and Sec63p might interact during translocation to activate the translocation apparatus.

Other hsc70 molecules are also involved in protein translo-
cations. In *S. cerevisiae*, the hsc70s are encoded by at least eight genes, including a subfamily known as the SSA genes (Werner-Wasburne 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). PpEF translated in a yeast extract can be posttranslationally translocated into yeast microsomes (Hansen et al., 1986; Rothblatt and Meyer, 1986; Waters andBlobel, 1986). The inefficient translocation of wheat germ–translated pppEF can be corrected by the addition of Ssalp or Ssa2p (Chirico et al., 1988; Deshaies et al., 1988). Cytosolic hsc70s 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 description 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* prepore protein 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 andBlobel, 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 (MATa, ura3-52, leu2-3, -112, pep4::URA3, N. Peyer, this laboratory), RSY151 (MATa, sec63-1, ura3-52, leu2-3, -112, pep4::URA3, N. Peyer, this laboratory), RSY156 (MATa, ura3-52, leu2-3, -112, pep4::URA3, Rothblatt et al., 1989), M1W141 (MATa, ura3-52, leu2-3, -112, rpl10, his3-11, -15, ssal::HIS3, ssal::LEU2, ssal::URA3, pGAL-SsA1-TRPI); Deshaies et al., 1988), and M1137 (MATa, kar2-199, ura3-52, leu2-3, -112, ade2-101, Vogel et al., 1989). Antibodies to SEC63p (Stirling et al., 1992), and Sec63p (Deshaies and Schekman, 1990) have been described, and antibodies to Sec33p were generated against a protein A–Sec33 fusion protein (Feldhahn 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 mem-
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 were layered onto a 55% sucrose solution containing the vesicles in a 5 ml ultracentrifuge tube (13 x 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 sensitivity 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 ferrocyanide. 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), 0.1% 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 MFW41 yeast cells (Deshayes et al., 1988) were grown at 30°C to a final OD600 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 (Chapelle 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 loaded onto 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 OD600 of cells was 1.1 mg of Ssalp as assessed by 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 B. The pellet was resuspended in 50 ml of buffer B 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 B, the salt concentration was increased to 0.75 M KCl, and the column was washed with another 20 ml. A 40-ml linear gradient to 2 M KCl was applied, 1-ml fractions were collected, and Kar2p was detected by immunoblottting with an anti-Kar2p antibody (Rose et al., 1989). Kar2p eluted from Q-Sepharose at ~0.6 M KCl. 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 of Translocation 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 35S-ppcF prepared from a yeast in vitro translation reaction, ppcF is translocated into microsomes. The signal peptide is then cleaved and the pro-a-factor (ppcF) becomes triply glycosylated (3gpcF), 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 ppcF is degraded, while a portion of the 3gpcF is protected (Fig. 1 B, lane 2). The 3gpcF 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 ppcF. To visualize the conversion of ppcF to ppcF by yeast signal peptidase (Böhm 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 ppcF, but not 3gpcF was visible (Fig. 1 A, lane 2). In this gel system, ppcF migrates more slowly than ppcF, even though it is shorter by 19 amino acids (Waters et al., 1988).

Figure 1. Comparison of ppcF 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 assay for translocation of 35S-ppcF. 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 ATP toS (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 azoleatin (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 two-fold 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 35S-pppF generated trypsin-protected poF 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 poF was membrane protected. The species that migrated at the position of 3gppF in the reaction with the reconstituted vesicles (Fig. 1 C, lane 1) probably was aggregated pppF, because higher molecular weight oligomers of pppF were also evident on this gel (not shown). Quantitation by Phosphorlmager analysis showed that the formation of protease protected poF was stimulated 3.5-fold by ATP, and the overall efficiency of the reaction (the amount of ATP-dependent protease-protected poF 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 poF, the amount of protease-protected pppF 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 pppF 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 pppF on the surface of the phospholipid vesicles. Given this anomalous behavior of a fraction of the pppF, the formation of protease-protected poF 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, Sec62p, and Sec63p contained 13% wt/wt sucrose.

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 poF in each gradient was quantified on a Phosphorlmager. 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 peF. Reconstituted vesicles were incubated in a translocation reaction with or without ATP and membranes were exposed to trypsin to remove any untranslocated, accessible pepF, 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 pepF 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 pepF 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 unilamellar and multi-lamellar structures.

**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 pepF, the periplasmic enzyme acid phosphatase, and the vacuolar enzyme carboxypeptidase Y (Rothblatt et al., 1989). The temperature-sensitive defect in pepF 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 pepF, 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 pepF 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 pepF (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
Cytosolic hsc70 (Ssalp in yeast) stimulates the translocation of ppoF 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 ppoF 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 ppoF 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 showing protease-resistant ppoF and pF; (bottom) quantitation of the amount of protease-protected pF from the upper image. Wild-type level (absolute translocation and processing efficiency of ~10%; see text for details) is taken to represent 100% translocation.

al., 1992), and was also completely restrictive in the reconstituted vesicles (Fig. 4). The reason for this discrepancy is unknown. Possibly, the sec63-I 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 pF 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 luminal 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.
from kar2-159 cells are unable to translocate pp~F, 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 pp~F 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 in Pelham, 1986; Ellis, 1987; Rothman, 1989; Gething and Sambrook, 1992). The KAR2 and SSAI 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 pp~F 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 luminaly oriented domain of Sec63p that has significant homology to the NH2 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 luminal surface of the translocation complex through an association with Sec63p. Conversely, a distinct cytosolic dnaJ homolog in yeast such as Ydj1p 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 pp~F 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 pp~F and for cotranslationally translocated molecules such as invertase (Rotheblatt 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 luminal 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; Brandage et al., 1990), and good progress is being made with a reconstituted protein translocation reaction from dog pancreas ER (Nicchitta et al., 1991; Görlach et al., 1992). Preliminary results demonstrate that a highly purified fraction of Sec63p restores translocation activity to proteoliposomes reconstituted from sec63 mutant mem-

Figure 7. Ssalp rescues the wheat germ-translated pp~F defect in microsomes. Wild-type microsomes and wheat germ-translation pp~F 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 3gp~F signal present when 25 μg of Ssalp was added to a reaction with wheat germ-translation pp~F and microsomes. (B) Proteins were added to the reaction mixture as follows: Buffer, 0 μg; 1, 2, 3, or 25 μg Ssalp. (C) Translocation assays were performed 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 3gp~F signal present when 25 μg of Ssalp was added to a reaction with wheat germ-translation pp~F and microsomes. (B) Proteins were added to the reaction mixture as follows: Buffer, 0 μg; 1, 2, 3, or 25 μg Ssalp.
branes. Similar biochemical complementation is anticipated as a means to assay and purify functional forms of Sec61p and Sec62p.

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References

Böhm, P. C., R. J. Deshaies, and R. Schekman. 1988. SEC11 is required for peptide signal processing and yeast cell growth. J. Cell Biol. 106:1035-1042.

Brandl, L., J. P. Hendrick, E. Schiebel, J. M. Driessen, and W. Wickner. 1990. The purified E. coli integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. Cell. 62:649-657.

Bulleid, N. J., and R. B. Freedman. 1988. Defective co-translational formation of disulfide bonds in protein disulphide-isomerase-deficient microsomes. Nature (Lond.). 335:642-651.

Caplan, A. J., and M. G. Douglas. 1991. Characterization of YDJ1: A yeast homologue of the bacterial dnak protein. J. Cell Biol. 114:609-621.

Chirico, W. J., M. G. Waters, and G. Blobel. 1986. 70K heat shock-related proteins stimulate protein translocation into microsomes. Nature (Lond.). 332:805-810.

Crook, E., B. Guthrie, S. Lecker, R. Lill, and W. Wickner. 1988. ProOmpA is stabilized by either purified E. coli target factor or canine signal recognition particle. Cell. 54:1003-1011.

Deshaies, R. J., and R. Schekman. 1987. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. J. Cell Biol. 105:635-643.

Deshaies, R. J., and R. Schekman. 1989. SEC62 encodes a putative membrane protein required for protein translocation into the endoplasmic reticulum. J. Cell Biol. 109:2653-2664.

Deshaies, R. J., and R. Schekman. 1990. Structural and functional dissection of Sec62p, an membrane-bound component of the yeast endoplasmic reticulum protein import machinery. Mol. Cell. Biol. 10:6024-6034.

Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature (Lond.). 332:800-805.

Deshaies, R. J., S. L. Sanders, D. Feldheim, and R. Schekman. 1991. Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex. Nature (Lond.). 349:806-808.

Driessen, A. J. M., and W. Wickner. 1990. Solubilization and functional reconstitution of the protein-translocation enzymes of Escherichia coli. Proc. Natl. Acad. Sci. USA. 87:1107-1311.

Ellis, J. 1987. Proteins as molecular chaperones. Nature (Lond.). 328:378-379.

Feldheim, D., J. Rothblatt, and R. Schekman. 1992. Topology and functional domains of Sec63p, an ER membrane protein required for secretory protein translocation. Mol. Cell. Biol. 12:3282-3286.

Flynn, G. C., T. G. Chappell, and J. E. Rothman. 1989. Peptide binding and release by proteins implicated as catalysts of protein assembly. Science (Wash. D.C.). 245:385-390.

Flynn, G. C., J. Pohl, M. T. Flocco, and J. E. Rothman. 1991. Peptide-binding specificity of the molecular chaperone BiP. Nature (Lond.). 353:726-730.

Franzusoff, A. J., and V. Cirillo. 1983. Solubilization and reconstitution of the glucose transport system from Saccharomyces cerevisiae. Biochim. Biophys. Acta. 724:153-159.

Gething, M.-J., and J. Sambrook. 1992. Protein folding in the cell. Nature (Lond.). 355:33-45.