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The Role of Molecular Chaperones in Protein Transport into the Endoplasmic Reticulum [and Discussion]

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The role of molecular chaperones in protein transport into the endoplasmic reticulum

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SUMMARY

In eukaryotic cells export of the vast majority of newly synthesized secretory proteins is initiated at the level of the membrane of the endoplasmic reticulum (microsomal membrane). The precursors of secretory proteins are not transported across the microsomal membrane in their native state. Typically, signal peptides in the precursor proteins are involved in preserving the transport-competent state. Furthermore, there are two alternatively acting mechanisms involved in preserving transport competence in the cytosol. The first mechanism involves two ribonucleoparticles (ribosome and signal recognition particle) and their receptors on the microsomal surface and requires the hydrolysis of GTP. The second mechanism does not involve ribonucleoparticles and their receptors but depends on the hydrolysis of ATP and on *cis*-acting molecular chaperones, such as heat shock cognate protein 70 (hsc 70). In both mechanisms a translocase in the microsomal membrane mediates protein translocation. This translocase includes a signal peptide receptor on the *cis*-side of the microsomal membrane and a component that also depends on the hydrolysis of ATP. At least in certain cases, an additional nucleoside triphosphate-requiring step is involved which is related to the *trans*-acting molecular chaperone BiP.

1. MECHANISMS INVOLVED IN PROTEIN TRANSPORT INTO MICROSOMES

All polypeptides have a unique intra- or extracellular location where they fulfil their function. In eukaryotes most proteins are synthesized in a single compartment, the cytosol. Thus, non-cytosolic proteins have to be directed to different subcellular locations. For such proteins, the sites of synthesis and functional location are separated by at least one biological membrane. Therefore, mechanisms exist to ensure the specific transport of proteins across membranes. In protein export one can distinguish between signal peptide-independent mechanisms and those in which a signal sequence is a prerequisite for specific transport (reviewed by Klappa *et al.* (1992)). The signal peptide-dependent mechanism (operating for export of the vast majority of newly synthesized proteins) is initiated at the level of the membrane of the endoplasmic reticulum (ER). This mechanism includes various steps such as association of the precursor protein with the ER membrane, membrane insertion, and, in the case of soluble proteins, completion of translocation. Precursor proteins are not transported in their native (i.e. folded) state, and signal peptides in the precursor proteins are involved in preserving the transport-competent (i.e. non-native) state as well as in facilitating membrane specificity. There are two alternatively acting mechanisms preserving transport competence in the cytosol (Wiech *et al.* 1991). In the first mechanism protein synthesis is slowed down. This mechanism involves two ribonucleoparticles, i.e. the ribosome and signal recognition particle (SRP), and their receptors on the microsomal surface, i.e. ribosome and SRP receptors (docking protein). This mechanism requires the hydrolysis of GTP (Connolly & Gilmore 1989; Connolly *et al.* 1991; Rapiejko & Gilmore 1992). To a certain extent, the SRP can be regarded as a molecular chaperone because it prevents the loss of transport-competence but is only transiently involved in the overall transport process (Ellis & van der Vies 1991). In the second mechanism protein folding or aggregation are slowed down. This mechanism does not involve ribonucleoparticles and their receptors, but depends on the hydrolysis of ATP and on molecular chaperones (see below). In both mechanisms a translocase in the microsomal membrane mediates protein translocation. This translocase includes a signal peptide receptor on the *cis*-side of the microsomal membrane, and a component that facilitates membrane insertion and also depends on the hydrolysis of ATP (Klappa *et al.* 1991). At least in certain cases, yet another nucleoside triphosphate-requiring step is involved which is related to the *trans*-acting molecular chaperone BiP.

2. COMPONENTS INVOLVED IN PROTEIN TRANSPORT INTO MICROSOMES

In the mammalian system the ribonucleoparticle-dependent pathway has been characterized in great

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detail. It involves SRP (Walter & Blobel 1981*a*,*b*; Zopf *et al.* 1990; Römisch *et al.* 1990; Lütcke *et al.* 1992), its receptor in the microsomal membrane (docking protein) (Meyer & Dobberstein 1980*a*,*b*; Lauffer *et al.* 1985; Tajima *et al.* 1986), and the ribosome (Perara *et al.* 1986) and its receptor. In the ribonucleoparticle-independent pathway ribonucleoparticles and their receptors are not involved. This independence was demonstrated by the observation that small precursor proteins, like preprocecropin A (ppcecA), are efficiently transported into salt-washed or trypsinized microsomes, i.e. in the absence of SRP or docking protein (Schlenstedt *et al.* 1990). Instead, several molecular chaperones preserve transport competence. One *cis*-acting chaperone has been identified as hsc 70 (Wiech *et al.*, 1987; Zimmermann *et al.* 1988). In addition, at least one *N*-ethylmaleimide-sensitive protein from the eukaryotic cytosol participates in this pathway (Wiech *et al.* 1993).

A similar situation exists in yeast (Chirico *et al.* 1988). The idea that hsc 70 is involved in the physiological process of transport of proteins into the endoplasmic reticulum is supported by data from genetic manipulations in yeast cells. Depletion of cytosolic hsp 70 proteins *in vivo* was observed to cause the accumulation of presecretory proteins (Deshaies *et al.* 1988). However, there also is ribonucleoparticle-dependent protein transport in yeast (Poritz *et al.* 1988; Ribes *et al.* 1988; Hann *et al.* 1989; Hann & Walter 1991; Amaya *et al.* 1990). The SRP54 protein was first identified as a homologue of mammalian SRP54 protein. Furthermore, the sec65 protein was identified genetically as a transport component, and according to the sequence analysis contains a domain with striking similarity to mammalian SRP19 protein (Stirling & Hewitt 1992; Hann *et al.* 1992). Genetic evidence suggested that the membrane proteins sec61, sec62, and sec63 are essential for transport (Deshaies & Schekman 1987, 1989; Sadler *et al.* 1989; Toyn *et al.* 1988). The sec63 protein contains a lumenal domain with striking similarity to bacterial DnaJ protein, a protein which is known to interact with DnaK, the bacterial hsp 70 homologue (Sadler *et al.* 1989). Furthermore, the trans-acting molecular chaperone BiP (KAR2 gene product) has been shown to have a direct role in transport (Vogel *et al.* 1990; Nguyen *et al.* 1991; Sanders *et al.* 1992).

3. THE ROLE OF MOLECULAR CHAPERONES IN PROTEIN RENATURATION AND IN PROTEIN TRANSPORT INTO MICROSOMES

Many of the molecular chaperones which have been identified so far belong to one of three protein families, i.e. the hsp 60, hsp 70, and hsp 90 families of stress proteins. To gain insight into the specificities of these three protein families, we set out to isolate members of these stress protein families from eukaryotic organisms and to compare their functions in two different assay systems (figure 1). One assay system (figure 1*a*), developed by U. Jakob and J. Buchner, deals with the role of stress proteins in the renaturation of denatured

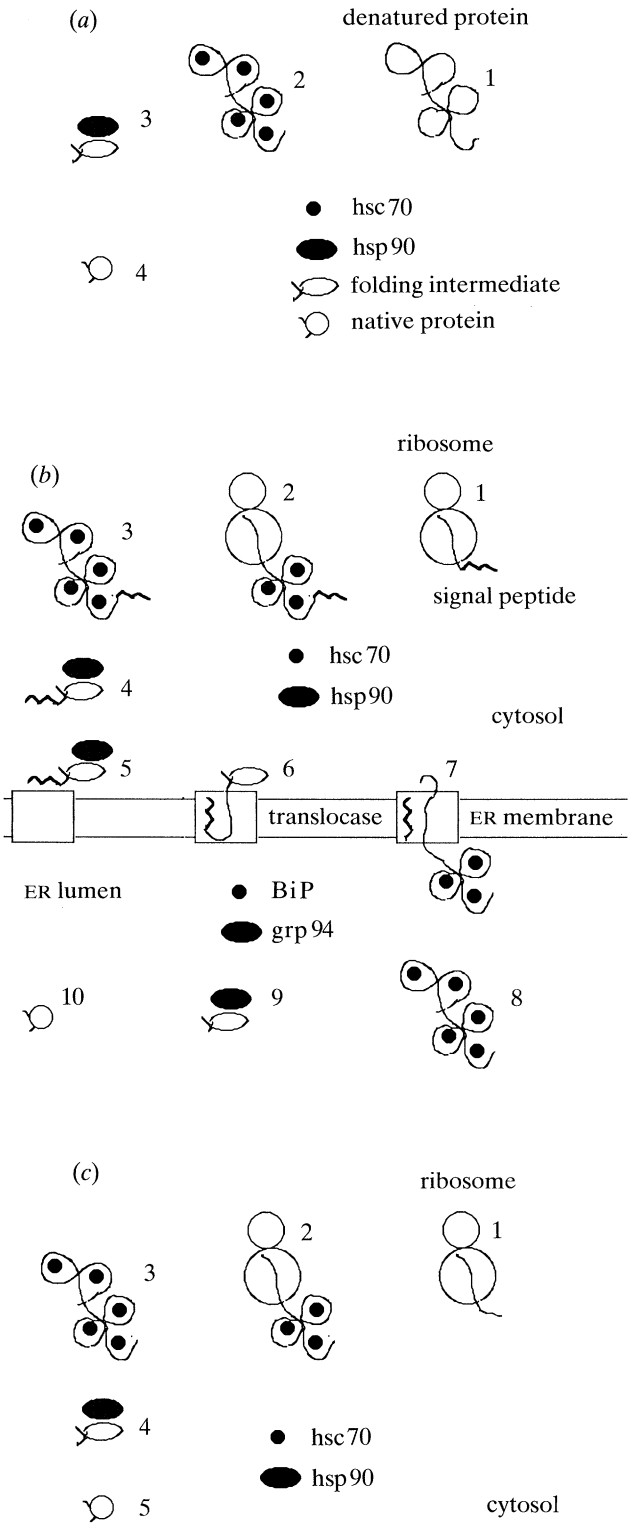

Figure 1. Working model for the role of molecular chaperones in transport of presecretory proteins into the endoplasmic reticulum and in protein folding. (*a*) The role of molecular chaperones in protein renaturation. (*b*) The role of molecular chaperones during synthesis of presecretory proteins in the cytosol and during ribonucleoparticle-independent translocation into the ER. (*c*) The role of molecular chaperones during the synthesis of cytosolic proteins. Symbols: hsc 70 = heat shock cognate protein 70; hsp 90 = heat shock protein 90; BiP = immunoglobulin heavy chain binding protein; grp 94 = glucose-regulated protein 94. The numbers indicate the order of the different stages.

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proteins (Buchner *et al.* 1991; Wiech *et al.* 1992). The other assay system (figure 1*b*) involves newly synthesized precursors of non-cytosolic proteins, and is concerned with the role of stress proteins in preserving the so-called transport-competent state (Wiech *et al.* 1987; Zimmermann *et al.* 1988). Our aim is to understand the role of molecular chaperones in both *in vitro* protein folding and during *in vitro* protein transport.

(*a*) *Protein renaturation*

Besides the 70 kDa heat shock proteins (hsp 70 protein family), eukaryotic organisms constitutively express stress proteins with a molecular mass of 90 kDa (hsp 90 protein family). Cytosolic hsp 90 may constitute up to two percent of the total cytosolic protein and has been shown to be involved in a variety of reactions. Hsp 90 was shown to associate with newly synthesized steroid hormone receptors, supporting their folding (Dalman *et al.* 1989). Furthermore, hsp 90 was found to form a complex with the newly synthesized plasma membrane protein $pp60^{v\text{-}src}$ prior to its assembly into the plasma membrane (Courtneidge & Bishop 1982). On the basis of these experiments hsp 90 was proposed to act as a molecular chaperone, at least in specific aspects of protein action. We argue that if hsp 90 is a molecular chaperone, not only involved in stabilizing a subset of cellular proteins in a certain conformation, it should be able to recognize and bind to non-native proteins, thereby influencing their folding to the native state. To test this hypothesis we studied the refolding, after denaturation, of a model protein (citrate synthase) in the presence and absence of hsp 90 purified from bovine pancreas (Wiech *et al.* 1992). Hsp 90 suppressed the formation of protein aggregates by binding to the target protein at a stoichiometry of one hsp 90 per one substrate molecule. Furthermore, at this ratio the yield of correctly folded and functional protein was increased significantly. Strikingly, the action of hsp 90 did not depend on nucleoside triphosphates. Thus, hsp 90 has a general folding activity; so far we were unable to demonstrate any effect of hsc 70 under similar conditions (Buchner *et al.* 1991).

(*b*) *Protein transport*

In our previous studies on protein transport into microsomes M13 procoat protein, the precursor of a bacterial plasma membrane protein, was used as a model protein (Wiech *et al.* 1987; Zimmermann *et al.* 1988). Procoat protein is exquisitely suited for this purpose because its transport into dog pancreas microsomes does not involve the ATP-requiring microsomal protein (Klappa *et al.* 1991). Thus, the only ATP-dependent reaction in this assay is related to the activity of molecular chaperones. Procoat protein is not efficiently transported into microsomal membranes after synthesis in a bacterial extract. However, the proportion of transported procoat protein is increased by, and shows a linear dependence on (i) purified hsc 70, and (ii) a *N*-ethylmaleimide-sensitive component present in a lysate of rabbit reticulocytes (Zimmermann *et al.* 1988). So far we have not detected any role of hsp 90 under these conditions.

(*c*) *Protein transport: revisited*

Towards the establishment of a transport reaction with purified components we recently addressed the question as to whether chemically synthesized preprocecropinA (ppcecA) can be transported into dog pancreas microsomes. For radiolabelling the HPLC-purified precursor was derivatized on lysine residues with either ^{14}C-methyl groups or ^{35}S-labelling reagent. When the radiolabelled ppcecA was precipitated with trichloroacetic acid in the presence of bovine serum albumin, subsequently solubilized in dimethyl sulphoxide and then incubated with microsomes (for details see figure 2), both membrane insertion (assayed as processing by signal peptidase) and transport of ppcecA (assayed as protease resistance of pcecA) occurred. On the other hand, chemically synthesized pcecA (i.e. lacking signal peptide), after radiolabelling and solubilization in dimethyl sulphoxide, was not transported. Furthermore, pcecA did not even become attached to the microsomes (figure 2). Thus we conclude that transport of ppcecA into dog pancreas microsomes depends on the signal peptide. In other transport systems solubilization of the precursor proteins by denaturants, and subsequent dilution into an aqueous solution, were found to circumvent the need for molecular chaperones to preserve transport competence (reviewed by Wiech *et al.* (1990)). Therefore, we asked whether in our system solubiliza-

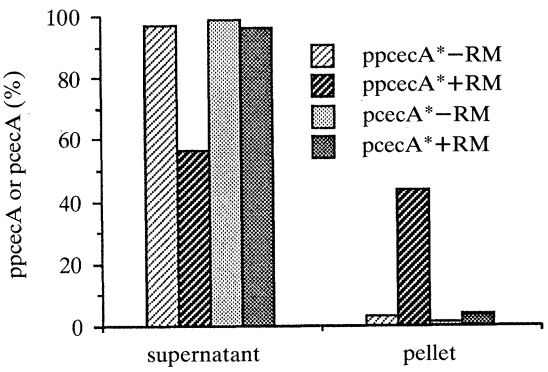

Figure 2. Binding of ppcecA and pcecA to dog pancreas microsomes. Following precipitation with trichloroacetic acid in the presence of bovine serum albumin, and solubilization in dimethyl sulphoxide, ^{14}C-labelled ppcecA (500 ng solubilized in 0.2 µl) and ^{14}C-labelled pcecA (500 ng solubilized in 0.2 µl), respectively, were added to 20 µl of transport buffer (Klappa *et al.* 1991) which did not contain ATP. The mixtures were divided into two aliquots and the aliquots were supplemented with either RM buffer (Klappa *et al.* 1991) or microsomes (RM: final concentration of microsomal protein: 1 mg ml^{-1}). After an incubation for 20 min at 37°C each mixture was subjected to subfractionation at a pH value of 7 as described previously (Schlenstedt *et al.* 1990). The samples were analysed by gel electrophoresis and fluorography. The amounts of ppcecA and pcecA, respectively, in the various fractions were quantified by laser densitometry of the fluorograph.

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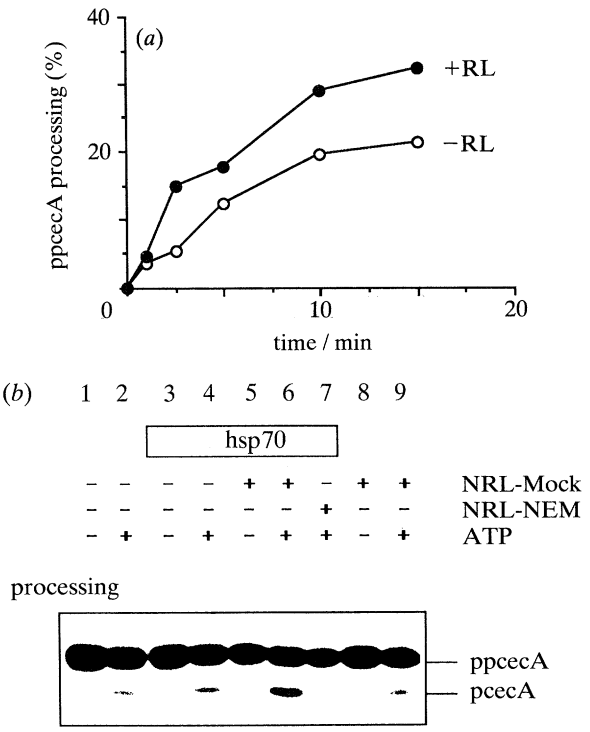

Figure 3. The role of hsc 70 in transport of ppecA into microsomes. (*a*) Following precipitation with trichloroacetic acid in the presence of bovine serum albumin and solubilization in dimethyl sulphoxide, ^{35}S-labelled ppecA (80 ng solubilized in 0.88 µl) was added to either 80 µl of rabbit reticulocyte lysate translation mix (RL) or 80 µl of transport buffer which contained ATP. The two mixtures were supplemented with microsomes (final concentration of microsomal protein: 1 mg ml^{-1}) and each one was divided into eight aliquots (final volume: 11 µl). Incubation of the various aliquots at 37°C was carried out for the times indicated. The samples were analysed by gel electrophoresis and fluorography. The amounts of ppecA and pcecA were quantified by laser densitometry of the fluorograph, and the efficiencies of processing of ppecA (given as pcecA in percent of ppecA plus pcecA) were plotted against the incubation time. (*b*) After precipitation with trichloroacetic acid in the absence of bovine serum albumin and solubilization in dimethyl sulphoxide, ^{14}C-labelled ppecA (4 µg solubilized in 0.88 µl) was added to 80 µl of transport buffer which contained microsomes (final concentration of microsomal protein: 3.5 mg ml^{-1}). The mixture was divided into nine aliquots (final volume: 50 µl) and the aliquots were supplemented with ATP, purified hsc 70 (200 µg ml^{-1}), an ammonium sulphate fraction derived from reticulocyte lysate (NRL) or NRL which had been pretreated with *N*-ethylmaleimide (NEM). RL refers to reticulocyte lysate. NRL-Mock refers to an ammonium sulphate cut derived from reticulocyte lysate which was mock treated, i.e. which was incubated with dithiothreitol (50 mM) plus NEM (10 mM) for 30 min at 25°C. NRL-NEM refers to the same ammonium sulphate cut. In this case, however, the protein solution was incubated first with NEM for 20 min and subsequently with DTT for 10 min. Incubation of the various aliquots at 37°C was carried out for 15 min. The samples were analyzed by gel electrophoresis and fluorography.

tion of ppecA in dimethyl sulphoxide had a similar effect. We observed that both membrane insertion and transport of ppecA occurred in the absence of reticulocyte lysate; however, the presence of lysate had a stimulatory effect (figure 3*a*). The stimulatory effect of the lysate was more pronounced when the precipitation with trichloroacetic acid was carried out in the absence of albumin (not shown). Next we asked whether the effect of lysate was due to hsc 70, the additional *N*-ethylmaleimide-sensitive component and ATP, as we had observed for procoat protein. There was little transport of ppecA in the absence of lysate or in the presence of limiting amounts of lysate (figure 3*b*). The transport efficiency, however, was increased when hsc 70 was added together with lysate; furthermore, the lysate was sensitive to pretreatment with *N*-ethylmaleimide. Note that after precipitation with trichloroacetic acid in the absence of albumin the transport efficiency reached the level shown in figure 3*a*, i.e. when albumin-coprecipitated ppecA was used, only after addition of hsc 70 plus lysate (figure 3*b*). We conclude from these data that molecular chaperones are not essential for, but can stimulate, transport of purified ppecA into microsomes. The observed ATP dependence, however, cannot be interpreted unequivocally, since ppecA transport involves translocase with its ATP-dependent component.

By employing chemically synthesized ppecA we were able to demonstrate that there is a microsomal component which is limiting for membrane insertion, and that this component is sensitive to photoaffinity labeling with 8-azido-ATP (Klappa *et al.* 1991). Studies on protein transport into yeast microsomes have suggested a role for BiP in protein transport across the yeast microsomal membrane (Vogel *et al.* 1990; Sanders *et al.* 1992). Although BiP had been shown not to be a component limiting for transport of *in vitro*-synthesized precursor proteins (i.e. at nM concentrations) into mammalian microsomes (Yu *et al.* 1989; Zimmerman & Walter, 1990; Nicchitta & Blobel, 1990), we asked whether BiP becomes limiting for transport of 100- to 1000-fold higher concentrations of a purified precursor protein, such as ppecA, and, therefore, was responsible for the observed azido-ATP effect. Microsomes were depleted of their lumenal proteins by treatment with increasing concentrations of octyl glucoside as described by Zimmerman & Walter (1990). When a concentration of 22.5 mM octyl glucoside was used during the pretreatment of microsomes, more than 90% of BiP was removed. However, after removal of the detergent the activity of the resealed microsomes with respect to processing of ppecA was unaffected. Because it is unlikely that photoaffinity labelling of microsomes with azido-ATP led to more than 90% derivatization of BiP, it seemed reasonable to conclude that photoaffinity labelling of BiP was not responsible for the observed inactivation of microsomes by photoaffinity labelling with azido-ATP (Klappa *et al.* 1991).

4. MODEL FOR RIBONUCLEOPARTICLE-INDEPENDENT TRANSPORT OF PROTEINS INTO MICROSOMES

There are consecutive steps of nucleotide hydrolysis involved in protein transport into the ER. In ribonucleoparticle-independent transport there is a first ATP-

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dependent reaction involved in preserving transport competence (related to hsc 70, figure 1*b*, stage 3 to 4) and a second one in facilitating membrane insertion (related to translocase, stage 5 to 6). At least in certain cases there may be a third step on the *trans*-side (related to BiP, stage 8 to 9). To ensure that precursor proteins do not stably fold before being transported into the endoplasmic reticulum both signal peptides in the precursors and molecular chaperones preserve the transport-competent state (most likely a molten globular state) (Wiech *et al.* 1990). We assume that the presence of a signal peptide on a nascent polypeptide chain or a freshly synthesized protein extends the half-life of the protein–chaperone complex compared to the half-life in the absence of a signal peptide (figure 1*b* versus 1*c*). Since hsc 70 and hsp 90 were observed to cooperate in hormone receptor function (Dalman *et al.* 1989), we proposed that they may be the molecular chaperones involved in protein transport (Wiech *et al.* 1991). However, a combination of these two molecular chaperones failed to stimulate in our assay. Therefore, we believe that additional components like DnaJ (and, possibly, a GrpE homologue) are required. We find this to be an attractive hypothesis because in the microsomal lumen a similar set of molecular chaperones, i.e. BiP (a member of the hsp 70 family), grp 94 (a member of the hsp 90 family) and a DnaJ homologue (sec63 protein), is present which is assumed to be involved in protein folding or transport. It is noteworthy in this context that cytosolic DnaJ homologues recently have been identified genetically in yeast cells (Caplan & Douglas 1991; Luke *et al.* 1991). An alternative candidate for interacting with hsc 70 is TCP1, which is found in the human, murine, yeast and rabbit reticulocyte cytosol, and which shares chaperonin activity with the other members of the hsp 60 protein family (Gao *et al.* 1992; Lewis *et al.* 1992; Yaffe *et al.* 1992).

Membrane association of the precursor proteins occurs via a putative signal peptide receptor (figure 1*b*, stage 4 to 5). With the help of the translocase the signal peptides are inserted into the membrane, most likely in form of a loop structure which is made up by the signal peptide plus the aminoterminus of the mature part (stage 5 to 6). The ATP hydrolysis at the microsomal level seems to be directly providing the energy for membrane insertion. For translocation to progress, the protein on the *cis*-side has to unfold completely (stage 6 to 7). The question is: where does the energy for unfolding come from? The energy for complete unfolding of a precursor protein may be as low as 10 kcal mol^{-1}. Thus, the initial hydrolysis of one ATP molecule would be sufficient to drive such an unfolding reaction. It is tempting to speculate that binding of the polypeptide chain, as it emerges in the lumen of the ER, to the *trans*-acting molecular chaperone BiP may provide the additional energy which is required for completion of translocation. Alternatively, translocation may be driven by spontaneous refolding on the *trans*-side of the target membrane. In either case, the energy gained by inter- or intramolecular interactions in the lumen would drive unfolding on the opposite side of the membrane.

In future experiments protein folding will have to be directly studied in the *in vitro* translation system (figure 1*c*). The proposal that the presence of a signal peptide on a nascent polypeptide chain or a freshly synthesized protein extends the half-life of the protein bound to molecular chaperones, as compared with the half-life in the absence of a signal peptide is one of the questions which will be addressed under these conditions (figure 1*b* versus 1*c*). Another question will be directed towards the similarities or differences in the role of molecular chaperones in both protein renaturation and protein folding during protein synthesis (figure 1*a* versus 1*c*). Furthermore, transport reactions employing reconstituted membrane vesicles (Nicchitta & Blobel 1990; Nicchitta *et al.* 1991), with a manipulated lumenal content, will have to be performed in order to assess the proposal on the role of BiP and grp 94 (figure 1*b*).

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Discussion

S. Lindquist (*Howard Hughes Medical Institute, University of Chicago, U.S.A.*). I would like to comment on the suggestion that hsp 90 might be involved in protein translocation. We have made some temperature-sensitive yeast strains which have limited amounts of hsp 90. We see no accumulation of protein precursors when these strains are exposed to nonpermissive temperatures and there are no differences in the competence of extracts in protein translocation assays *in vitro*. Is it possible to explain the authors' results on the basis that the hsp 90 is binding to hsp 70 and hence removing its function rather than competing with hsp 70?

R. Zimmermann. This explanation is possible, but there is another observation which suggests that this is not the case. The DnaK protein from *E. coli* cannot substitute for hsp 70 in this system, nor can it compete with hsp 70; but the GroEL protein, especially if combined with GroES, has the same effect as hsp 90 and competes with hsp 70. Because hsp 90 has a similar effect on protein refolding as the GroE system, we suspect that we are looking at the same phenomenon in respect to the competition. But we cannot exclude that the competition by hsp 90 is as Professor Lindquist suggests, and the competition by GroE is something different.

W. J. Welch (*Department of Medicine and Physiology, University of California, San Fransciso, U.S.A.*). Workers in the steroid hormone field believe that hsp 90 interacts with the hydrophobic domain necessary for dimerization of the steroid hormone receptor, so this may be relevant to your observations. Do the authors still observe the 150 kDa lumenal protein if the column is washed with high salt before elution with ATP?

R. Zimmermann. Yes.

P. Viitanen (*Du Pont de Nemours, Wilmington, U.S.A.*). Have the authors studied the effects of immunodepletion of hsp 90 from the rabbit reticulocyte lysate to determine whether hsp 70 is also depleted, and have you established that the prevention of aggregation of unfolded citrate synthase by hsp 90 is affected by *N*-ethyl maleimide?

R. Zimmermann. The immunodepletion experiment did not work. In the citrate synthase renaturation experiments, hsc 70 competes with hsp 90, but we have not studied the effect of *N*-ethyl maleimide on hsp 90.

W. J. Welch. Have the authors shown that precursor proteins interact directly with hsp 73 in the rabbit reticulocyte lysate in the absence of ATP?

R. Zimmermann. Adding hsp 70 to the procoat protein in the absence of ATP renders it resistant to protease attack; addition of ATP makes this protein sensitive to added protease. The hsp 70 antisera we have tried do not recognize native hsp 70 so we have been unable to try coimmunoprecipitation experiments. We have observed an interaction of hsp 70 with procoat protein on native gel electrophoresis.

G. H. Lorimer (*Du Pont de Nemours, Wilmington, U.S.A.*). Do the authors know how many different polypeptides become labelled with azido-ATP in the microsomal extract, and can they protect them against this labelling with ATP? Can one take the ATP eluate from the ATP agarose column and selectively label one protein with azido-ATP?

R. Zimmermann. At a low concentration of labelled azido-ATP about ten polypeptide bands become labelled and this labelling is prevented by ATP. Three of these bands occur in the ATP eluate and one of these is the BiP protein. At high concentrations of azido-ATP (e.g. 5 mM) many more polypeptides become labelled, but it is clear that not all the polypeptides in the microsomal extract are labelled at concentrations of azido-ATP where effects on protein transport can be observed.

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