Protein Folding before and after Translocation into the Yeast Endoplasmic Reticulum

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ABBREVIATIONS

A alanine
ATP adenosine triphosphate
BiP immunoglobulin heavy chain-binding protein
CHX cycloheximide
CPY carboxy peptidase Y
D aspartic acid
DTT dithithreitol
E glutamic acid
ER endoplasmic reticulum
GDP guanosine diphosphate
GTP guanosine triphosphate
Hsp heat shock protein
KDa kilodalton
MHC major histocompatibility complex
NAC nascent-chain associated complex
NGFR<sub>e</sub> nerve growth factor receptor ectodomain
PAGE polyacrylamide gel electrophoresis
PDI protein disulfide isomerase
PPI peptidyl-prolyl cis/trans isomerase
RAMP4 ribosome-associated membrane protein 4
SDS sodium dodecyl sulphate
SRP signal recognition particle
TRAM translocating chain-associating protein
Ub ubiquitin
UGGT UDP-glucose:glycoprotein glucosyltransferase
UPRE unfolded protein response element
SUMMARY

Translocation of secretory proteins to the endoplasmic reticulum (ER) is the first step in the secretory pathway. Artificial reporter proteins were used to study posttranslational translocation and folding in Saccharomyces cerevisiae cells. E. coli β-lactamase and rat nerve growth factor receptor ectodomain (NGFRε) were fused to the Hsp150Δ-carrier, an N-terminal fragment of a yeast secretory glycoprotein, whose signal peptide was found to confer posttranslational translocation. β-lactamase mutants binding to penicillin G in an irreversible or reversible fashion were created and fused to the carrier. β-lactamase is a tight globular protein and NGFRε consists of four separate extended domains. All fusion proteins were found to be translocated posttranslationally and properly folded in the yeast ER and thereafter secreted to the culture medium.

The β-lactamase portion of the fusion protein was found to adopt a native-like conformation in the yeast cytosol before it was translocated into the ER. Similar to the authentic protein, it was trypsin-resistant. Furthermore, it had similar catalytic activity and $K_m$ values for nitrocefin on both sides of the ER membrane. The folded cytoplasmic form of the fusion protein could be chased to the ER and to the culture medium in active conformation. In contrast to the requirement of a disulfide bond for activity in the ER, productive folding in the cytoplasm was not dependent on disulfide bond formation, highlighting the differences of the cytosol and the ER lumen as folding milieus. Translocation of the mutant β-lactamase fusion protein was found to be prevented when it was locked to a stable conformation due to the irreversibly bound penicillin G. The prefolded β-lactamase fusion protein was attached to the translocation sites and unfolded before insertion into the translocation pore was initiated. The results of this study show for the first time that a polypeptide is folded in the yeast cytoplasm before translocation into the ER. They reveal a novel function in the cytoplasm, unfolding of prefolded protein, resulting in signalling for pore opening.

In the ER lumen the chaperone BiP assists in translocation and folding of newly synthesised polypeptides. The fusion proteins Hsp150Δ-β-lactamase and Hsp150Δ-NGFRε were used to study if these two functions were independent. Translocation of Hsp150Δ-β-lactamase and Hsp150Δ-NGFRε were dependent on functional BiP. However, only the β-lactamase portion required BiP for conformational maturation in the ER. Thus, the requirement for BiP for folding is apparently substrate-specific, and some proteins, such as NGFRε, do not need to be assisted by BiP.
INTRODUCTION

1. Overview

The eukaryotic cell contains membrane-bound organelles that perform highly specialized functions. Each organelle has specific proteins that define its structure and function. The maintenance of these compartments requires that newly synthesized proteins are accurately targeted to their final destination.

All polypeptides destined for transport through the secretory pathway begin their journey by crossing the endoplasmic reticulum (ER) membrane. After translocation into the ER, proteins are transported to the Golgi apparatus and from there to the lysosome or the vacuole in yeast, the plasma membrane, or the exterior of the cell, unless they are meant to stay in the ER or Golgi. All transport steps after translocation into the ER occur by vesicular budding and fusion, and translocation across membranes is no longer required. The secretory pathway of yeast *Saccharomyces cerevisiae* has been defined by temperature-sensitive secretion mutants, known as sec mutants, in which the transport of proteins is reversibly blocked into different organelles of the pathway (Figure 1).

In the special folding environment of the ER, proteins are modified co- and posttranslationally, and acquire their mature tertiary and quaternary structures. Even though the information needed for proper folding resides in the amino acid sequence of the polypeptides, many proteins assist and accelerate folding. If proper maturation fails, the aberrant products are retained in the ER and eventually degraded. Thus, in addition to being a special folding milieu, secretory proteins are sorted in the ER from resident proteins and incorrectly folded proteins, which if secreted could be harmful or even dangerous to the cell.

Figure 1. A schematic picture of the secretory pathway of *S. cerevisiae* presenting the Sec proteins mentioned in this study.
2. Protein translocation

2.1. Protein targeting to the endoplasmic reticulum

2.1.1. Signal peptides

In eukaryotic cells, most proteins are synthesized on free ribosomes in the cytoplasm. They find their destination by specific targeting sequences or signal peptides. Signal peptides of secretory proteins are usually N-terminal extensions (Blobel and Dobberstein, 1975), but can also be located within a protein or at its C-terminal end (von Heijne, 1990).

Signal peptides vary in their amino acid composition and length, but they all contain a positively charged N-terminus of one to five residues, a central hydrophobic core of six to fifteen amino acid residues and a polar region of three to seven amino acids. Studies with signal peptide mutants revealed that the hydrophobic core region is the most essential part required for targeting (von Heijne, 1985). The polar region contains a recognition site for signal peptidases (von Heijne, 1990), which are enzymes known to cut the signal peptide, once its targeting function has been completed. The cleavage occurs either during translocation or soon after completion of translocation. The yeast signal peptidase is a heterotetrameric protein complex whose enzymatic activity is provided by the subunits Sec11p (Böhni et al., 1988; YaDeau et al., 1991) and Spc3p (Fang et al., 1997; Meyer and Hartmann, 1997). In bacteria, a similar translocation system resides in the plasma membrane (Hartmann et al., 1994). The crystal structure of the bacterial signal peptidase has been resolved (Paetzelt et al., 1998). The active site of the peptidase is surrounded by an extended hydrophobic patch suggested to position the active site near the membrane, where it meets the cleavage site of the translocating substrate. In the case of membrane protein translocation, the signal peptide can anchor the protein in the membrane, instead of being cleaved off (High and Dobberstein, 1992).

Signal peptides can direct proteins to the ER membrane through different targeting pathways. In yeast, translocation into the ER can occur either during protein synthesis, i.e. cotranslationally, or after protein synthesis and release from the ribosome, i.e. posttranslationally. The hydrophobicity of the signal peptide determines which translocation pathway is used (Ng et al., 1996). Signal peptides interact with many proteins during translocation, indicating that they are not merely lipophilic peptides, but play an active role during protein translocation into the ER. Mothes et al. (1998) reported that all steps of cotranslational translocation, including the recognition of signal peptide, can be reproduced with purified translocation components in detergent solution in the absence of lipids, indicating that signal peptides are ultimately recognized by protein-protein interactions.

2.1.2. Cotranslational translocation

As secretory proteins emerge from the ribosome during translation, the signal recognition particle (SRP), a complex of six polypeptides (72, 68, 54, 19, 14, 9 kD) and an RNA component, binds to the signal peptide (Walter et al., 1981). In addition to its ability to bind to signal peptides, SRP was shown to cause a site-specific arrest in chain elongation that was released by
binding to microsomal membranes (Walter and Blobel, 1981; Wolin and Walter, 1989). The arrest of protein synthesis gives time for the nascent-chain ribosome complex to find a binding site in the ER membrane before domains of the polypeptide are exposed in the cytosol, which could lead to their folding, misfolding or aggregation.

Analysis of the protein components of SRP has identified subcomplexes responsible for the above-mentioned functions. SRP54 protein binds to the signal sequence (Krieg et al., 1986; Kurzchalia et al., 1986). It has a methionine-rich domain, which is responsible for the binding of signal peptide (High and Dobberstein, 1991). Zheng and Nicchitta (1999) showed that a leucine-rich signal peptide is necessary for optimal interaction with SRP and proposed that SRP maintains the signal peptide in a conformation competent for binding to the membrane. The crystal structure of the signal peptide binding subunit of the SRP from *Thermus aquaticus* shows a deep groove bounded by a flexible loop and lined with hydrophobic residues (Keenan et al., 1998). As a flexible, hydrophobic environment, the groove can accommodate signal peptides of different lengths and primary structures. Matoba and Ogrydziak (1998) reported that factors other than hydrophobicity, such as conformation or orientation of the signal peptide, can affect its interaction with SRP. The elongation arrest is caused by the SRP9 and SRP14 subcomplexes (Siegel and Walter, 1985), and targeting to the ER membrane requires SRP68 and SRP72 subunits (Siegel and Walter, 1988).

The nascent chain-ribosome complex is targeted to the ER translocation machinery via the interaction of SRP with the SRP receptor in the ER membrane (Gilmore et al., 1982; Meyer et al., 1982). Binding of GTP to SRP54 and to the SRP receptor stabilizes the SRP-SRP receptor complex (Bacher et al., 1996; Rapiejko and Gilmore, 1997) and leads to dissociation of SRP from the nascent chain and the ribosome (Connolly and Gilmore, 1989). Hydrolysis of GTP releases the SRP from the receptor (Connolly et al., 1991) and prepares it for the next targeting round.

Homologues of mammalian SRP components have been identified in yeast. Yeast Srp54p, a homologue of mammalian SRP54 protein, was found to be in a complex with a major yeast cytoplasmic RNA, scR1, and to be important for targeting of proteins to the ER (Hann and Walter, 1991). Stirling et al. (1992) isolated a temperature-sensitive yeast mutant, sec65-1, as being defective in membrane protein insertion. Yeast Sec65p is a homologue of SRP19 (Stirling and Hewitt, 1992), and it was shown to be required for the stable association of Srp54p with SRP and for the recycling of Srp54p (Hann et al., 1992; Regnaq et al., 1998). Brown et al. (1994) purified the yeast SRP and found that it contains homologues of mammalian SRP14, SRP68 and SRP72. Lack of any of the SRP components led to slow cell growth and inefficient protein translocation into the ER. The yeast SRP contains a protein component, Srp21p, which appears to have no counterpart in mammalian SRP.

The fidelity of the cotranslational targeting of a polypeptide is thought to be maintained by the nascent polypeptide-associated complex (NAC). NAC is an abundant heterodimeric protein complex that interacts with both cytoplasmic and secretory nascent chains as they emerge from the ribosome (Wiedman et al., 1994). In the absence of NAC, any ribosome was able to bind to the ER membrane and even cytoplasmic proteins without signal peptides could be translocated across the membrane. Thus, it was proposed that
NAC may be a negative regulator for ER targeting. However, Neuhof et al. (1998) reported that SRP-independent targeting of the ribosome to the ER membrane occurs in the absence or presence of NAC, indicating that NAC may have another role. In the absence of SRP, binding of ribosome-nascent chain complex to the ER membrane was dependent on free Sec61p, which is a component of the translocation channel, and nontranslating ribosomes were found to compete for this interaction. In the presence of SRP, this competition was not observed. Thus, the binding of SRP to ribosome-nascent chain complexes gives a competitive advantage in the interaction with ER membrane. Signal peptide-bound SRP has been shown to occupy the membrane attachment site on ribosomes and is therefore thought to inhibit the binding of NAC (Möller et al., 1998).

2.1.3. Posttranslational translocation

In mammalian cells, most proteins are translocated cotranslationally, but short peptides and some proteins are able to translocate posttranslationally without SRP (Schlenstedt et al., 1990). In yeast, a larger number of proteins are translocated posttranslationally. As a fast-growing organism, its translation rate may exceed the translocation rate (Matlack et al., 1998). Yeast cells are viable in the absence of SRP (Hann and Walter, 1991; Brown et al., 1994). Furthermore, cells lacking SRP adapt over time and thereby gain the ability to translocate proteins, which are translocated cotranslationally under normal conditions (Ogg et al., 1992). The time dependence of adaptation suggests that synthesis of specific proteins may be required. Indeed, upon the depletion of SRP54, the cytoplasmic levels of both cytoplasmic Ssa1p chaperone and Ydj1p cochaperone were increased (Arnold and Wittrup, 1994), although this could also be a general stress response caused by the accumulation of secretory protein precursors in the cytoplasm.

The targeting step of posttranslational translocation is apparently SRP-independent, but requires a signal peptide. Ng et al. (1996) demonstrated that the hydrophobic core of the signal peptide determines which of the two pathways is used in yeast. The more hydrophobic the core, the more tightly it is bound to SRP. Less hydrophobic sequences do not bind to SRP, and these preproteins use the posttranslational route.

2.2. The translocation channel

Genetic studies in yeast led to the identification of Sec61p, a multispanning integral membrane protein of the ER required for translocation of both secretory and membrane proteins and proposed to form a channel for translocation (Deshaiies and Schekman, 1987; Stirling et al., 1992; Wilkinson et al., 1996). Sec61p interacts directly with Sss1p (Esnault et al., 1993; Wilkinson et al., 1997) and Sbh1p (Panzner et al., 1995), with these three proteins forming the Sec61p complex (Figure 2). Plath et al. (1998) proposed that Sss1p acts as a surrogate signal peptide, which is replaced by the arrival of the signal peptide. Yeast cells have homologues of Sec61p and Sbh1p, these being Ssh1p and Sbh2p, respectively (Finke et al., 1996; Toikkanen et al., 1996), which form a second trimer together with Sss1p in the ER membrane. Because it does not associate with an additional protein complex required for posttranslational translocation, this complex is assumed to
function solely in the cotranslational pathway (Finke et al., 1996).

Homologues of all these proteins have been found in mammalian cells and they can even function in yeast (Hartmann et al., 1994). The mammalian homologue of Sbh1p, Sec61β, facilitates cotranslational translocation and interacts with the signal peptidase (Kalies et al., 1998). Interestingly, the interaction of Sec61β with the subunit of signal peptidase complex was dependent on the presence of membrane-bound ribosomes, suggesting that these interactions are enhanced when translocation is initiated. Sec61β has been shown to be phosphorylated, and phosphorylation moderately stimulated protein translocation into the ER (Gruss et al., 1999). Phosphorylation might regulate interactions between the channel and other proteins involved in translocation.

In mammalian cells, a translocating chain-associated membrane protein (TRAM) is involved early in translocation of polypeptides (Görlich et al., 1992a). By site-specific crosslinking, High et al. (1993) showed that the very N-terminal region of the signal peptide is in contact with TRAM, and the hydrophobic core of the signal peptide is in contact with Sec61p. TRAM was suggested to regulate the cytosolic exposure of secretory proteins during the translocational pause (Hedge et al., 1998). Yeast cells lack a homologue of TRAM.

Sec61 protein is the receptor for the ribosome in the ER membrane (Figure 2). Görlich et al. (1992b) isolated from mammalian ER a membrane protein bound to ribosomes, which was a homologue of Sec61p. Similar to the yeast protein, it was located in the immediate vicinity of nascent polypeptides during their membrane passage, since they could be cross-linked to it (Mothes et al., 1994). Sec61p was protease-resistant in the presence of ribosomes (Kalies et al., 1994). A three-dimensional image reconstruction of a ribosome bound to the yeast Sec61 complex revealed an alignment of the

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Figure 2. Co- and posttranslational protein translocation in yeast. In cotranslational translocation the Sec61 complex mediates protein import into the ER. Ribosome binds tightly to the channel thereby sealing it from the cytoplasmic side. In posttranslational translocation the Sec61 complex associates with the Sec62/63 complex to form a heptameric complex. The lumenal ATP:ase BiP/Kar2p interacts with the Sec63p and provides the energy for translocation.
translocation channel with the site on the large ribosomal unit which is believed to be the exit site for the nascent polypeptide (Beckman et al., 1997). As the protein is elongated, it has only one way out, and therefore, protein synthesis drives translocation and no other energy supply is needed.

Electron microscopy studies verified that the Sec61p complex forms a channel (Hanein et al., 1996). Purified complexes from mammalian and yeast cells formed ring-like structures in detergent with a pore diameter of about 20Å. Each pore consisted of three or four Sec61p complexes. Similar ring structures were also seen in reconstituted and native membranes. The addition of ribosomes was seen to increase the number of ring structures of Sec61p complexes in reconstituted membranes (Hanein et al., 1996). The nascent chain is presumably inserted into the channel in a loop structure (Shaw et al., 1988) (Figure 2). Song et al. (2000) noted that in the absence of a functional Sec61 complex, the signal sequence could not be released from the SRP54 protein. They proposed that the GTP hydrolysis cycle of SRP and its receptor complex is regulated by the Sec61 complex. The hydrophobic portion of the signal peptide has been cross-linked to lipids (Martoglio et al., 1995), indicating that the channel is open laterally to lipids. Thus, hydrophobic portions of translocating polypeptides could reside in a hydrophobic environment, while the hydrophilic portions would slide through the aqueous parts of the channel. Based on the measurements of the minimal length of the translocating polypeptide required to bridge the distance between the ribosomal peptidyl transferase site in the membrane-bound ribosomes and the luminal active site of oligosaccharyl transferase, Whitley et al. (1996) suggested that non-hydrophobic nascent chains adopt a fully extended conformation during passage through the translocon, whereas the hydrophobic sequence appeared to form a helical structure when located in the channel.

Since the ER membrane encloses a special folding milieu, it is important to maintain impermeability to even small molecules. The translocation channel must open to translocate proteins and close when the work is done. The model of cotranslational translocation favoured the assumption of a narrow channel, but Hamman et al. (1997) demonstrated with quenching agents of different sizes that the actual size of the channel could expand up to 60 Å. However, because the channel is impermeable even to ions, the opening and closing of the channel must be tightly regulated. Crowley et al. (1993) first illustrated the existence of a seal by fluorescent quenching studies, in which they showed that small molecules from the cytoplasmic side of the membrane could not reach translocating polypeptides carrying fluorescent probes. The tight junction of the ribosome and Sec61p forms the seal to the cytoplasmic side of the channel. Jungnickel and Rapoport (1995) presented a model in which the signal peptide of a nascent polypeptide is recognized twice, first in the cytoplasm by SRP, and a second time inside the membrane by the ER translocation machinery, Sec61p. The initial interaction between the ribosome-nascent chain complex and the Sec61p complex is weak; it is sensitive to high salt concentrations, and the nascent chain remains accessible to added proteases. Once the nascent polypeptide has reached a critical length, the ribosome is bound more tightly to the Sec61p channel, and the polypeptide is no longer accessible to proteases, indicating its insertion into the Sec61p channel. In reconstituted bacterial membranes,
addition of signal peptides to the cytoplasmic site of the membrane led to the opening of the translocation channels (Simon and Blobel, 1992). The transition from weak to strong binding involves the recognition of the signal peptide, and it occurs at the same nascent chain length as the opening of the channel (Jungnickel and Rapoport, 1995). The aqueous translocon pore is closed to the ER lumen until the nascent chain reaches a length of seventy amino acids (Crowley et al., 1994). BiP, a lumenal chaperone, seals the channel from the luminal side of the membrane before and early in translocation (Hamman et al., 1998). The closure of the channel requires dissociation of the ribosome into its subunits (Simon and Blobel, 1991). Wang and Dobberstein (1999) characterized oligomeric complexes involved in translocation across the ER membrane and could not detect any size differences between the unengaged Sec61p complex and the ribosome-bound one, suggesting that the Sec61p complex does not disassemble into its subunits after completion of translocation. Taken together, the translocation channel seems to be a very dynamic structure.

In posttranslational translocation, the Sec61p complex associates with the tetrameric Sec62/63p complex forming a heptameric complex (Deshaises et al., 1991; Panzner et al., 1995) (Figure 2). The complete heptameric complex is required for posttranslational translocation into reconstituted proteoliposomes. Sec62p and Sec63p are essential membrane proteins, which span the membrane two and three times, respectively (reviewed in Corsi and Schekman, 1996). The two other components, Sec71p and Sec72p, are not essential for cell growth. Sec71 is a single-spanning membrane protein, and Sec72 is a peripherally associated protein on the cytosolic side of the ER membrane (Deshaises et al., 1991; Green et al., 1992). A Sec63p homologue has been found in mammalian cells, and it resides in the ER membrane covered with ribosomes (Skowronek et al., 1999). The tetrameric subcomplex has been thought to replace the functions of ribosomes and SRP. Sec62p, Sec71p and Sec72p have all been proposed to play a role in the recognition and binding of signal peptide (Müsch et al., 1992; Feldheim and Schekman, 1994) (Figure 2). The obvious transient nature of the interaction between the signal peptide and its receptors makes it difficult to study in vivo. Dünnwald et al. (1999) used the split-ubiquitin technique to detect a possible interaction between Sec62p and the signal peptide of pre-pro-α-factor. In this technique, the N- and C-terminal halves of ubiquitin (Ub) are fused to two test proteins, which are expected to interact. If they do, Ub is reconstituted in cells from the two halves, and can thereafter be cleaved by Ub-specific proteases, which are present in all eukaryotic cells. This results in release of the reporter protein which was fused to the C-terminal portion of Ub. The release of the reporter serves as a readout indicating reconstitution of Ub. By this method Sec62p was shown to interact with the signal peptide in living cells. Like the ribosome, the Sec62/63p complex stimulates the assembly of Sec61p complex rings (Hanein et al., 1996).

Plath et al. (1998) studied the recognition of signal peptide in posttranslational translocation. By cross-linking experiments, they observed that the signal peptide interacts with Sec61p independently of ATP and Kar2p, a lumenal chaperone known to be required for posttranslational translocation. They found that the two transmembrane domains of Sec61p were primarily responsible for the interaction with the hydrophobic core of the signal peptide. The signal peptide adopts
a helical structure within the channel and is likely to be oriented perpendicularly to the plane of the membrane. Cross-links between the signal peptide and lipids were also detected while the polypeptide resided in the channel, indicating that the signal peptide binding site is located at an interface between the channel and lipids. Matlack et al. (1997) reported that posttranslational translocation could be observed in detergent solution without membranes. Binding of a precursor protein was dependent on functional signal peptide and both Sec61 and Sec62/63 complexes.

2.3. Chaperones in translocation

Molecular chaperones are defined as molecules that prevent protein aggregation and facilitate folding by maintaining polypeptides in productive folding pathways (Gething and Sambrook, 1992). The 70 kDa class of heat shock proteins are involved in translocation both on the cytoplasmic and the lumenal side of the ER membrane. They are ATPases that bind and release hydrophobic peptides (Bukau and Horwich, 1998). In yeast, cytosolic Hsp70s involved in translocation are the Ssa1-4 proteins (Deshaies et al., 1988), and the ER luminal Hsp70s are Kar2p (Vogel et al., 1990), and its homologue Lhs1p, which facilitates translocation of a subset of proteins into the ER (Craven et al., 1996).

Deshaies et al. (1988) studied the effect of cytoplasmic Hsp70s on posttranslational protein translocation in vivo. They used a yeast strain in which all the SSA1-4 genes were disrupted, and Ssa1p was introduced to the cells in a plasmid under a galactose-regulated promotor. When the cells were grown on glucose, the level of Ssa1p decreased with concomitant cytoplasmic accumulation of ER-targeted preproteins. The requirement of Ssa1p for posttranslational translocation could be bypassed if the preprotein was denatured (Chirico et al., 1988). The conclusion from these studies was that the cytoplasmic Hsp70s keep the polypeptide in a translocation-competent or unfolded conformation.

The ATPase activity of the Hsp70s is enhanced by DnaJ chaperones. Ydj1p is a cytosolic yeast homologue of DnaJ (Caplan and Douglas, 1991). The temperature-sensitive mutant of Ydj1p is unable to stimulate the ATPase activity of the Ssa1p (Caplan et al., 1992), which is required for protein release (Cyr et al., 1992). Despite the fact that BiP and Ssa1p are 63% identical, they are unable to substitute for one another during posttranslational translocation (Brodsky et al., 1993). This was demonstrated to be caused by specific interactions with unique DnaJ homologues (McCellan et al., 1998).

Since elongation and translocation of polypeptide chains are uncoupled in posttranslational translocation, additional energy is needed to drive the translocating chain across the membrane. The movement of the polypeptide through the translocation channel requires the presence of Kar2p or its mammalian counterpart BiP (Vogel et al., 1990). Translocation is dependent on the interactions of BiP/Kar2p with the luminal J-domain of Sec63p (Sanders et al., 1992; Brodsky and Schekman, 1993; Scidmore et al., 1993; Lyman and Schekman, 1995; Corsi and Schekman, 1997; Matlack et al., 1997). Each J-domain activates several BiP/Kar2p molecules to trap neighbouring peptides with low sequence specificity (Misselwitz et al., 1998). BiP appears to interact with the J-domain only very transiently, but this brief interaction is
sufficient to induce the hydrolysis of ATP and to activate BiP for peptide binding. In the absence of peptides, BiP binds to its J-partner, indicating that BiP does not wait for the substrate while bound to the J-domain (Misselwitz et al., 1999).

Two models have been proposed by which BiP/Kar2p and the J-domain of Sec63p could provide the driving force for posttranslational translocation. In one, BiP/Kar2p has an active role in stimulating the forward sliding of polypeptide by pulling the polypeptide through the channel (Glick, 1995). Alternatively, BiP/Kar2p could act as a molecular ratchet (Simon et al., 1992) by binding to the translocating polypeptide, thereby preventing its backward movement. Matlack et al. (1999) showed that bound BiP does minimize backward movement of the polypeptide through the channel. In fact, antibodies against the polypeptide could replace BiP, indicating that a ratchet is sufficient to achieve translocation. However, this does not exclude the possibility of BiP functioning in the pulling reaction as well. Additional force may be required if the polypeptide is in a folded conformation on the cytosolic side of the membrane, or if cytosolic proteins are strongly bound to it.

Although the energy needed to drive cotranslational translocation is thought to be gained from coupling protein synthesis and translocation, mutations in BiP prevented not only posttranslational translocation but also cotranslational translocation (Brodsky et al., 1995). In the case of cotranslational translocation, translocation of the last 60 amino acids cannot be driven by elongation since they are buried in the ribosome and the translocaose. Thus, even in cotranslational translocation, completion of translocation may be dependent on chaperones. Indeed, Nicchitta and Blobel (1993) provided evidence that luminal components are required for net transfer of secretory proteins to mammalian microsomes. Depletion of the luminal contents of ER membranes by isolating them at pH 10 resulted in dramatic translocation defects in vitro. The translocating polypeptides were inserted across the membrane and were accessible to signal peptidase and oligosaccharide transferase, but remained free to pass through the membrane to the cytosolic side. The defect could be complemented by the addition of luminal proteins, indicating that luminal proteins prevent translocated polypeptides from sliding back to the cytosol.

BiP/Kar2p has been suggested to affect events on the cytosolic face of the ER membrane. Mutations in BiP prevented interaction between a polypeptide and the translocation channel (Sanders et al., 1992), illustrating that BiP functions early in translocation on the opposite face of the ER membrane. BiP appeared to activate the translocation complex. This was indeed observed in membrane-free translocation assays; BiP was necessary for the movement of preproteins from the recognition site of Sec61p to the channel as well as for their movement through the channel (Lyman and Schekman, 1997; Matlack et al., 1997).

2.4. Retrotranslocation

Translocation of proteins to the ER is not an irreversible process. The Sec61 channel is the channel for retrograde transport as well. This was first observed when MHC class I heavy chains in human cytomegalovirus-infected cells were transported from the ER to the cytosol (Wierz et al., 1996a). This process was shown in coimmunoprecipitation studies to
involve Sec61 complex, and to eventually lead to degradation of the heavy chains by the cytosolic proteasome (Wierz et al., 1996b). Misfolded secretory proteins in the ER are also re-exported to the cytosol in a Sec61p-dependent fashion (Pilon et al., 1997). Sec61p and Sss1p themselves are degraded in the cytosol by proteasome (Biederer et al., 1996), triggered by disassembly of the translocation complex. Like wild-type proteins, misfolded soluble proteins dissociate from the Sec61 channel after completion of translocation (Plemper et al., 1999a) and the signal peptide is removed (Hiller et al., 1996; Werner et al., 1996). This implies that the opening of the channel from the luminal side must be triggered by a mechanism that is different from that used during translocation into the ER.

A mutant form of carboxypeptidase Y (CPY), which is degraded by the proteasome under normal conditions, was found to be stabilized in the ER in sec63-1 and kar2-159 cells, suggesting that their respective proteins have a role in retrotranslocation (Plemper et al., 1997). However, ER protein export and import seem to be mechanistically distinct, since specific kar2 mutations that stabilize otherwise degradative substrates work well in inward translocation. In addition, defects in or depletion of Ssa1p had no effect on degradation (Brodsky et al., 1999). As in other translocation events, in retrotranslocation the channel seems to work in conjunction with other components, including ER membrane proteins Der1p, Der3p/Hrd1p and Hrd3p (Hampton et al., 1996; Knop et al., 1996; Bordallo et al., 1998). Based on genetic studies, Der3p/Hrd1p and Hrd3p functionally interact with each other and with Sec61p (Plemper et al., 1999b).

3. Protein maturation in the ER

Nascent polypeptides are modified in the ER lumen. Some modifications occur during translocation across the ER membrane. Signal peptides are cleaved by signal peptidase, the catalytic site of which resides in the ER lumen (Böhni et al., 1988; YaDeau et al., 1991; Fang et al., 1997; Meyer and Hartmann, 1997). Oligosaccharides are attached to selected residues (Abejon and Hirschberg, 1992; Kelleher et al., 1992), and disulfide bridges are formed by protein disulfide isomerases (Freedman, 1984). Oligomeric proteins are assembled in the ER. All these events affect protein folding, which is assisted by molecular chaperones and folding enzymes.

A hypothesis based on coimmunoprecipitation and cross-linking experiments postulates that ER-resident proteins interact loosely with each other to form a dynamic matrix (Sambrook, 1990). The lumen of the ER contains high levels of calcium ions which may mediate protein interactions. Indeed, many ER-resident proteins are calcium-binding proteins, and depletion of calcium has been shown to cause misfolding and aggregation (Lodish et al., 1992; Suzuki et al., 1991). The matrix of ER proteins could keep newly synthesized proteins associated to it until folding is completed and the affinity would dissipate (Tatu et al., 1995). Transmembrane proteins could keep the matrix in contact with membrane sites.
where translocation takes place, leaving other ER areas free to accumulate folded proteins ready for export.

3.1. Glycosylation

Oligosaccharides in the plasma membrane or cell wall and secreted glycoproteins are classified by the nature of their linkage to the polypeptide: the N-linked (asparagine amine) and O-linked (hydroxyl group of serine or threonine) glycans. The biosynthesis of N-glycans, especially the initial steps, is similar between species, but the biosynthesis of O-glycans is less conserved (Tanner and Lehle, 1987). Protein-bound oligosaccharides are beneficial during maturation, as they increase solubility of folding intermediates and stabilize the protein conformation. N-glycans also allow newly synthesized proteins to interact with the lectin-based chaperone system (Trombetta and Helenius, 1998).

3.1.1. N-glycosylation

N-linked glycosylation in eukaryotes involves two different processes: the assembly of the lipid-linked core oligosaccharide Glc₃Man₉GlcNac₂ at the ER membrane, and the en bloc transfer of the oligosaccharide core from the lipid dolichol pyrophosphate to selected asparagine residues of nascent polypeptides. In the assembly step, at least eleven different yeast gene products add fourteen sugars in a stepwise manner (Kukuruzinska et al., 1987). The transfer step is mediated by the oligosaccharyltransferase complex, which recognizes on the nascent polypeptide as an acceptor site the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline (Kukuruzinska et al., 1987). The core oligosaccharide attached to the polypeptide is modified in the ER lumen by removal of three glucose and one mannose residues by the action of glucosidases I and II and a mannosidase (Tanner and Lehle, 1987). The trimmed core oligosaccharide is then extended in the Golgi complex by addition of mannose residues, leading to the formation of two types of structures: the short- and high-mannose chains. The degree of protein glycosylation is controlled in cells, since not all potential glycosylation sites are utilized. Glycosylation may be competed by protein synthesis, translocation and folding kinetics. In mammalian cells, a ribosome-associated membrane protein, RAMP4, was reported to regulate N-glycosylation by translocational pausing (Schröder et al., 1999).

3.1.2. O-glycosylation

In yeast, O-glycosylation is initiated in the ER (Haselbeck and Tanner, 1983). Dolichol monophosphate is the donor of the first mannose residue transferred to the hydroxy groups of serine and threonine (Tanner and Lehle, 1987; Immervoll et al., 1995; Lussier et al., 1995). Although the consensus sequence for O-glycosylation in yeast has yet to be determined, a proline residue in the vicinity of serine or threonine may enhance the first mannosylation reaction. The elongation of the O-linked chain occurs in the Golgi. O-linked chains are unbranched and contain at most four to five mannosyl residues (Lehle and Bause, 1984).
3.2. Lectin-like chaperones: Calnexin and calreticulin

Calnexin couples N-glycosylation of newly synthesized proteins with their productive folding in the ER (Bergeron et al., 1994). Calnexin is an integral type I ER membrane protein, which was originally identified as a major calcium-binding protein of the mammalian ER (Wada et al., 1991). The substrate-binding domain resides in the lumen. The ER-localization signal RKPRRE resides at the C-terminus. Its cytoplasmic tail contains a phosphorylation site (Bergeron et al., 1994), and phosphorylation was shown to lead to increased association of calnexin with membrane-bound ribosomes (Chevet et al., 1999). Coimmunoprecipitation experiments in the presence of the N-glycosylation inhibitor tunicamycin demonstrated that calnexin binds to N-glycosylated proteins (Ou et al., 1993). The binding preference for glycoproteins is based on a lectin-like affinity for monoglucosylated N-linked oligosaccharides (Hammond et al., 1994). Moreover, calnexin has been shown in vitro to act solely as a lectin, since binding of monoglucosylated Rnase B –protein was independent of the conformation of the glycoprotein (Zapun et al., 1997). Calreticulin, a soluble luminal homologue of calnexin, was shown to bind similarly to unfolded glycoproteins (Ou et al., 1993). The on and off cycle would proceed until folding is complete. More recent in vivo studies with thermosensitive viral glycoprotein have supported this model (Cannon and Helenius, 1999).

The mannose residues of the N-linked sugars have been proposed to set a time limit for folding. Misfolded glycoproteins are retained in the ER by the UGGT-calnexin-glucosidase II cycle, which leads to the complete trimming by mannosidases (Helenius et al., 1997). Jakob et al. (1998) demonstrated that the number and linkage of mannose residues of an N-linked oligosaccharide determine the degradation of misfolded proteins. Deletion of mannosidases Mns1p, Alg9p and Alg12p led to reduced degradation of a mutated carboxy peptidase Y (CPY). It was speculated that a lectin-like receptor would recognize the trimmed oligosaccharide and together with a chaperone such as BiP would guide the misfolded protein to retrotranslocation and subsequent degradation in the cytosol by the proteasome machinery. Yeast KRE5 is a homologue of the mammalian UGGT (Meaden et al., 1990). However, no
glucosyltransferase activity for this yeast protein has been demonstrated so far (Jakob et al., 1998). However, monoglucosylated N-linked sugar chains of glycoproteins were found to reduce the level of unfolded protein in the ER under mild reducing conditions (Jakob et al., 1998).

In addition to its role in proofreading of glycoproteins, calnexin may have another function in mammalian cells. It has been reported to bind unglycosylated polypeptides (Arunachalam and Cresswell, 1995; Kim and Arvan, 1995). This was, however, suggested to be caused by aggregation (Cannon et al., 1996). Recently, calnexin was identified as a peptide-binding protein and postulated to have a role in the removal and degradation of peptides in the ER (Spee et al., 1999).

### 3.3. Classical chaperones

The nascent polypeptide encounters a number of molecular chaperones and folding enzymes upon entering the lumen of the ER and begins to fold, in some cases, even before translation is completed (Hammond et al., 1994). Among the most abundant and best-characterized chaperones in the ER is BiP. As an Hsp70 protein, it consists of two domains, a highly conserved N-terminal ATP-binding domain and a C-terminal peptide-binding domain. BiP associates transiently with numerous proteins during folding and binds more permanently to misfolded proteins and incompletely assembled oligomers (Gething and Sambrook, 1992). It was originally identified as a protein binding noncovalently to free immunoglobulin heavy chains (Haas and Wabl, 1983).

BiP binds to unfolded nascent polypeptides (Simons et al., 1995; Hendershot et al., 1996). In vitro binding studies with a set of peptides of random sequence but a defined chain length revealed that BiP recognizes heptapeptides of aliphatic residues (Flynn et al., 1991). The same kind of peptide characteristics for BiP binding were found by affinity screening of a peptide library expressed on the surface of bacteriophages (Blond-Elguindi et al., 1993). Furthermore, the aliphatic residues were preferred in an alternating manner, suggesting that the extended sequence, in which hydrophobic residues face the same direction, would fit into the peptide-binding pocket of BiP (Blond-Elguindi et al., 1993). The crystal structure of the peptide-binding domain of the bacterial Hsp70 homologue DnaK with a bound peptide has been determined. The peptide resides in the binding pocket in an extended conformation (Zhu et al., 1996). It has been speculated that aliphatic peptide residues would occur approximately every sixteenth residue in an average globular protein (Flynn et al., 1991), enabling the binding of BiP to the vast majority of secretory proteins. In spite of this, some secretory proteins do not associate with BiP in normal conditions (Morris et al., 1997).

Like all Hsp70 proteins, BiP has a high affinity for ATP but a low turnover number (Kassenbrock and Kelly, 1989). The binding and hydrolysis of ATP is coupled to peptide binding and release. ADP binding stabilizes peptide binding, whereas ATP binding induces conformational changes in the peptide-binding domain, causing the release of bound peptides (Palleros et al., 1993).

Yeast \textit{KAR2} encoding a homologue of BiP, was isolated as a gene required for nuclear fusion after conjugation of haploid cells and
shown to be essential for viability (Normington et al., 1989; Rose et al., 1989). Expression of both mammalian and yeast BiP is induced by accumulation of unfolded proteins in the ER (Gething and Sambrook, 1992; Rose et al., 1989). In addition, the expression of yeast KAR2 is regulated by heat shock (Rose et al., 1989; Mori et al., 1992; Kohno et al., 1993).

Yeast ER contains another Hsp70-related protein, Lhs1p (also called Cer1p or Ssi1p) (Baxter et al., 1996; Craven et al., 1996; Hamilton and Flynn, 1996). In addition to its role in facilitating translocation, it has been suggested to have chaperoning activity, particularly at lower temperatures (Hamilton et al., 1999). Saris et al. (1997; 1998) have shown that Lhs1p is required in yeast ER for refolding, stabilization and acquisition of secretion competence of heat-denatured proteins. Unlike BiP, Lhs1p is not essential under normal conditions, but is required for acquisition of thermotolerance (Saris et al., 1997). It appears to belong to a heat-resistant survival machinery enabling yeast cells to recover from severe heat stress (Jämsä et al., 1995b; Saris et al., 1997; 1998). Mammalian cells have another abundant ER protein, GRP94, which interacts transiently with unfolded proteins (Melnick et al., 1994). The function of this protein is thought to be similar to that of BiP.

3.4. Folding enzymes: Protein disulfide isomerases and peptidyl prolyl isomerases

Disulfide bonds formed in the reducing environment of ER lumen stabilize the three-dimensional structure of numerous secretory proteins and the quaternary structure of some protein complexes. Enzymes that catalyze the formation and/or rearrangement of these bonds are called folding enzymes.

Protein disulfide isomerase (PDI) belongs to the thioredoxin superfamily and catalyzes both the isomerization and formation of disulfide bonds (Freedman, 1984). As a highly abundant ER luminal protein, it constitutes nearly 1% of the total cellular proteins. It is highly conserved between species. Quite surprisingly, PDI has four thioredoxin-like folds, even though only two of the domains display sequence homology to thioredoxin (Kemmink et al., 1997). The protein has two catalytic sites, double cysteines, in two thioredoxin-related domains, which are separated by two similar domains lacking redox-active sites (Edman et al., 1985; Freedman et al., 1994; Kemmink et al., 1997). PDI has been isolated as a homodimer (Freedman et al., 1994). In yeast, PDI is essential for cell survival (Scherens et al., 1991), and its foremost function is to reorganize disulfide bonds of non-native proteins (Laboissiere et al., 1995).

The isomerase activity of PDI, where PDI is transiently bound via mixed disulfide to its substrate, resembles the function of chaperones in keeping the polypeptide in a folding-competent stage. The full isomerase activity of PDI requires the presence of domains lacking the catalytic site (Darby et al., 1998). Interestingly, one of these domains provides the core of peptide-binding ability to PDI (Klappa et al., 1998). An unfolded protein response element (UPRE) was found in the promoter of PDI, supporting PDI’s function as a chaperone (Shamu et al., 1994). The function of PDI has recently been linked to the quality control machinery; it has been shown to be required for export and degradation of a cysteine-free misfolded...
secretory protein from the endoplasmic reticulum (Gillece et al., 1999).

Oxidized glutathione was proposed to be the source of the oxidizing equivalents needed to generate disulfide bonds in the ER (Hwang et al., 1992). However, mutant yeast cells lacking glutathione were shown to be capable of efficient disulfide bond formation (Frand and Kaiser, 1998). Ero1p, a novel essential protein in the ER membrane, was demonstrated to be responsible for maintaining PDI in an oxidized state (Pollard et al., 1998; Frand and Kaiser, 1999).

Cooperation between different ER proteins creates additional functions. ERp57, a member of the protein disulfide isomerase family, has been cross-linked to partially deglucosylated glycoproteins (Oliver et al., 1997). Calnexin and calreticulin present glycoproteins lacking disulfides to ERp57, which catalyzes the formation of the disulfide bond, thus enhancing folding (Zapun et al., 1998). Interaction of both the substrate and the enzyme with the lectin brings them into close proximity, thus enhancing the catalytic activity of Erp57. Other PDI-like proteins may function similarly with other ER proteins.

During protein synthesis at the ribosomes most peptide bonds are connected in the trans conformation. The trans conformation is often remained in native protein structures. The peptidyl-prolyl bond, however, can exist in cis conformation as well. The isomerization reaction was rate-limiting in refolding experiments (Kiefhaber et al., 1990). Since the folding intermediates are sensitive to proteolytic digestion and aggregation, an enzyme catalyzing the reaction was proposed to exist. Peptidyl prolyl isomerases (PPIs) catalyze the isomerization of cis and trans peptide bonds on the N-terminal side of proline residues. These enzymes are found in all cellular compartments where protein folding occurs. PPIs are members of three families, the cyclophilins, the FK-binding proteins (FKBPs) and the parvulins (Rudd et al., 1995). Accumulation of unfolded protein in the yeast ER induces expression of FKBP FPR1 (Partaledis and Berlin, 1993), suggesting their role in protein folding in vivo.
AIMS OF THE STUDY

Translocation of secretory proteins into the endoplasmic reticulum initiates their journey through the secretory compartments to the extracellular milieu. In yeast, proteins are translocated into the ER either simultaneously with protein synthesis, cotranslationally, or after protein synthesis is completed, posttranslationally. More than two decades of research on cotranslational translocation has led to a detailed picture of this process, while pieces of the mechanism of posttranslational translocation are still missing. Translocating polypeptides encounter a number of molecular chaperones and folding enzymes on the lumenal side of the ER membrane. The aims of this study were to expand on the mechanism of posttranslational translocation in living Saccharomyces cerevisiae cells, with particular emphasis on the cytoplasmic events before the actual crossing of the membrane, and to study the role of molecular chaperones in translocation as well as in folding.

The specific aims were:

I: To study the conformational stage of secretory proteins on the cytosolic side of the ER membrane before their posttranslational translocation into the ER,

II: To determine, whether a folded secretory protein could be accommodated by the translocation channel and inserted into the ER, and

III: To study the role of ER-located chaperone BiP in folding of nascent polypeptides and to determine, whether its functions in translocation and folding are separate.
MATERIALS AND METHODS

The yeast strains used in this study are listed in Table 1, and the defects of mutants are described in Table 2. The used experimental methods are listed in Table 3. The detailed description of each method is found in the original publications. The reporter proteins are schematically presented in Figure 3.

Table 1. The yeast strains used in this study.

<table>
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Materials and methods

Table 2. Relevant defects of the mutant yeast strains.

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<td>Trafficking in Golgi</td>
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<td>Δerg6</td>
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Table 3. The methods used in this study.

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Figure 3. Schematic presentation of the reporter proteins used in this study. A) Hsp150Δ-β-lactamase, B) Δ1-18Hsp150Δ-β-lactamase and C) Hsp150Δ-NGFRe. The Hsp150Δ consists of subunit I (horizontally striped) and an N-terminal part of the subunit II composed of a repetitive region (11 white boxes) and part of a unique C-terminus (diagonally striped box). The signal peptides are indicated by black boxes, the cysteine residues by stars and the potential N-glycosylation site of NGFRe by N. An arrow indicates the site of mutation, in which glutamic acid 166 is replaced either with alanine or aspartic acid. Hsp150Δ contains 95 potential O-glycosylation sites.
Hsp150 is a yeast secretory glycoprotein (Russo et al., 1992) consisting of a cleavable signal peptide of 18 amino acids, subunit I and subunit II. The two subunits are separated by a Kex2 recognition site, which is presumably cleaved in the Golgi. Subunit II contains a region of 19 amino acids, which is repeated 11 times, and a unique C-terminal region containing four cysteines, which form at least one intrachain disulfide bond (Jämsä et al., 1994). The promoter of the HSP150 includes heat-inducible elements (Russo et al., 1992). An N-terminal fragment of Hsp150, Hsp150D, was used as a carrier in all fusion proteins in this study. It consists of the signal peptide for ER targeting, subunit I and the repetitive region of subunit II (Jämsä et al., 1995a). The carrier fragment has 95 potential O-glycosylation sites, but not a single N-glycosylation site (Russo et al., 1992). All the O-glycosylation sites of the first 53 amino acids of Hsp150 are utilized (Suntio et al. 1999). When fused to E. coli β-lactamase or the extracellular domain of rat nerve growth factor receptor (p75) (NGFR), it has been shown to confer secretion competence to both heterologous proteins (Simonen et al., 1994; 1996).

To study posttranslational protein translocation into the ER and folding in living yeast cells, four different Hsp150Δ fusion proteins were used. E. coli β-lactamase was chosen as a reporter, because it has enzymatic activity that reflects its conformation and is easy to determine. Furthermore, in its authentic form, it is trypsin-resistant and has a globular structure with a single disulfide bond near the active site (Jelsch et al., 1992). Two point-mutated β-lactamases having decreased deacylation reaction velocities with antibiotics (Adachi et al., 1991) also were fused to the Hsp150D carrier (II). The crystal structure of the mutant β-lactamase which irreversibly binds benzylpenicillin has been solved (Strynadka et al., 1992). Based on high sequence homology with the tumour necrosis factor receptor ectodomain (TNFR) (Radeke et al., 1987) and the crystal structure of TNFR (Banner et al., 1993), NGFR is likely to have an extended rod-like structure of four domains, each of which have three intradomain disulfide bonds (III, Figure 2). NGFR has one N-glycosylation site in the first domain, which is glycosylated in roughly half of the molecules in the yeast ER (Simonen et al., 1996). The high number of cysteines and disulfide bonds of NGFR were anticipated to challenge the folding apparatus in yeast ER.
2. Protein folding before translocation into the ER (I)

2.1. Hsp150\(\Delta\) is translocated posttranslationally (I)

According to the hydrophobicity plot of the signal peptide (Ng et al., 1996), Hsp150 was thought to use the posttranslational translocation pathway. This was verified by taking advantage of specific sec mutants having defects in either co- or posttranslational translocation. sec62-101 cells are constitutively defective in posttranslational translocation (Ng et al., 1996). The strain expressing Hsp150\(\Delta\)-\(\beta\)-lactamase was pulse-labelled with \(^{35}\)S methionine/cysteine and chased with cycloheximide (CHX). After immunoprecipitation and SDS-PAGE, no protein could be seen in the culture medium samples even after 120 minutes of chasing (I, Figure 1). Instead, a 66 kDa protein was detected in cell lysates. The O-glycosylated ER form of Hsp150\(\Delta\)-\(\beta\)-lactamase has been shown to migrate as a 110 kDa band in SDS-PAGE (Simonen et al., 1994). The smaller 66 kDa band was therefore likely to present the unglycosylated precursor of the fusion protein. In similarly labelled and chased wild-type and sec65-1 strains, Hsp150\(\Delta\)-\(\beta\)-lactamase was secreted into the culture medium. In the sec65-1 mutant, the cotranslational pathway is blocked at a temperature of 37°C due to the nonfunctional SRP (Stirling et al., 1992). Thus, the signal peptide of Hsp150 uses the posttranslational pathway to enter the ER lumen.

2.2. The translocation intermediate on the cytoplasmic face of the ER membrane (I)

To study the topology of the 66 kDa protein, experimental conditions were sought to retard the translocation process but not to totally inhibit it. As posttranslational translocation requires ATP (Vogel et al., 1990), a decrease of the ATP level was presumed to slow down translocation. Indeed, in labelling experiments at high cell densities, where the availability of glucose became limiting, more 66 kDa form could be detected than with low cell density samples (I, Figure 2). To confirm that the 66 kDa form was capable of translocating across the membrane, sec18-1 cells in which the fusion of secretory vesicles to the Golgi is prevented at the restrictive temperature were labelled and chased under high cell density conditions. During the chase, the 66 kDa form was converted to the glycosylated form of 110 kDa in 20 minutes (I, Figure 3A). The translocation of authentic Hsp150 was not retarded at high cell densities (I, Figure 3B), as the unglycosylated form of 50 kDa could hardly be detected even after a one-minute pulse (data not shown). Thus, the unglycosylated form of the fusion protein could be detected not only in cells having a translocational defect, but also as an intermediate form in cells which were wild-type for translocation.

To reveal the topology of the different forms of Hsp150\(\Delta\)-\(\beta\)-lactamase, microsomes were isolated after incubation of sec18-1 cells at the restrictive temperature under high cell density, and subjected to SDS-PAGE and Western blot analysis with anti-\(\beta\)-lactamase antiserum. Both the 66 kDa form and the ER form of 110 kDa could be detected (I, Figure 4). The 66 kDa form was
sensitive to trypsin digestion, with the concomitant increase of a 32 kDa protein fragment. When digestion with trypsin was done in the presence of Triton X-100 to solubilize the membranes, the 110 kDa form also disappeared and the immunoreactive 32 kDa band was detected. As the digestion of the 66 kDa form occurred in the absence of Triton X-100, it was likely to reside on the cytoplasmic side of the ER membrane. The appearance of the 32 kDa fragment suggested that the β-lactamase portion of the fusion protein on both sides of the ER membrane was probably folded, since the authentic protein has the same size and is known to be trypsin-resistant (Minsky et al., 1986). In contrast, the Hsp150 portion of the fusion protein was presumed to be trypsin-sensitive due to its many recognition sites and lack of a regular secondary structure (Jämsä et al., 1995a). Similar results were obtained when the digestion experiments were performed with metabolically labelled whole cell lysates (I, Figure 5).

2.3. The translocation intermediate is active (I)

To gain further information about the conformation of the β-lactamase portion of the cytoplasmic fusion protein, the activity of β-lactamase was measured in sec63-1 cells, where posttranslational translocation is blocked at the restrictive temperature (Rothblatt et al., 1989). At 37°C, activity accumulated inside the cells, whereas nothing could be detected in the medium (I, Figure 7 B). After addition of CHX, the cells were shifted to the permissive temperature of 24°C. The activity inside the cells decreased with time and was concomitantly secreted to the medium. The reversal of the sec63-1 block was not complete, as revealed by a pulse-labelling experiment (I, Figure 6). This explains the lower activity in the medium samples of sec63-1 cells as compared with sec18-1 cells after chasing at the permissive temperature. In contrast to the activity of β-lactamase in the ER lumen (I, Figure 7A), the cytoplasmic activity was unaffected by the reducing agent DTT (I, Figure 7B), which diffuses across the membranes and prevents the formation of disulfide bridges and reduces the existing ones (Jämsä et al., 1994). Thus, the reporter protein folded to a active conformation in the cytoplasm prior its translocation into the ER, probably even without the formation of the disulfide bond. This conclusion was supported by the similarity of Km values for nitrocefin of cytoplasmic and ER forms of Hsp150Δβ-lactamase to those of authentic E. coli β-lactamase (I, Table 2).

3. The conformation of proteins attached to the translocon (II)

3.1. The β-lactamase mutants (II)

Above I showed that the β-lactamase portion of newly synthesized Hsp150Δβ-lactamase folded first into a native-like conformation in the yeast cytoplasm and was thereafter translocated. The question thus arises, whether it passed the translocon in a folded form or whether it first had to be unfolded. β-lactamases hydrolyze β-lactam rings of antibiotics. The reaction occurs through an acyl-enzyme intermediate, in which the enzyme and the substrate are covalently bound to each
other. To find out whether the folded Hsp150Δ-β-lactamase had to unfold before translocation, we took advantage of two β-lactamase mutants which bind to antibiotics in an irreversible and reversible manner. In these mutants, glutamic acid Glu166, which resides at the bottom of the active site of β-lactamase, was mutated to alanine (E166A) or aspartic acid (E166D). A covalent, stable complex with the β-lactam antibiotic, benzylpenicillin (PenG), accumulates in E166A mutants, originally created for studying the catalytic mechanism of the hydrolysis reaction. However, PenG bound to the E166D mutant decays slowly and the hydrolytic activity is retained (Adachi et al., 1991).

3.2. The effect of PenG on the reporter (II)

The two mutant forms of β-lactamase were fused to the Hsp150Δ-carrier to create the fusion proteins E166A and E166D, and expressed in yeast strains, which are wild type for secretion. The ERG6 gene, whose product functions in sterol synthesis, was disrupted to change the sterol composition of membranes to allow the penetration of drugs (Jackson and Képès, 1994). The cells were preincubated with PenG and pulse-labelled with [35S]-methionine/cysteine. The majority of the fusion protein E166A was found in cell lysates in a cytoplasmic 66 kDa form (II, Figure 1A), and very little was in the medium. The cytosolic form did not disappear during the chase with CHX. In the absence of PenG, the E166A fusion protein was translocated and secreted normally, indicating that the mutation alone did not affect translocation of the fusion protein (II, Figure 1B). However, the mutation inactivated the fusion protein (II, Figure 2). Thus, PenG, by binding irreversibly to the mutated β-lactamase portion of the fusion protein, presumably prevented its unfolding and thereby its translocation into the ER.

3.3. Subcellular localization of PenG-bound reporter protein (II)

Next, we wanted to verify that the E166A fusion protein was on the correct export pathway. The cells harbouring the mutated fusion protein were incubated with PenG and then subjected to immunofluorescent staining either with β-lactamase antiserum or anti-Lhs1 antibody to mark the ER. These two antibodies revealed very similar structures (II, Figure 3A). In yeast, the ER mostly resides beneath the plasma membrane. E166A bound to the PenG was thus likely to reside on the ER membrane. The Hsp150Δ-β-lactamase lacking a signal peptide resided in the cytosol (II, Figure 3Ac), indicating that the association of E166A with the ER membrane was dependent on the signal peptide.

Hence, it follows that translocation channels should be saturated in vivo during the expression of the mutated fusion protein in the presence of PenG. To see whether this was the case, the translocation of ER-resident protein BiP was studied. Unlabelled E166A fusion protein was expressed in the presence of PenG at different times. Then, the cells were metabolically labelled and chased. The cell lysates were subjected to immunoprecipitation with anti-BiP antiserum and SDS-PAGE. The longer the accumulation of the unlabelled PenG-bound fusion protein, the more pre-BiP was detected and the less mature form was generated (II, Figure 4). The synthesis of pre-BiP was found to decrease concomitantly with the accumulation time of E166A fusion protein bound to PenG. Thus, the accumulation of
the unprocessed precursor form of the mutated fusion protein in the presence of PenG led to the saturation of translocation channels, demonstrating that it was bound to functional translocons. As BiP uses the cotranslational pathway to enter the ER lumen (Ng et al., 1996), the same Sec61p complexes seem to be used by substrates of both translocation pathways in vivo.

The extent of translocation of the mutated fusion protein was then studied. E166A was labelled in the presence of PenG and subjected to immunoprecipitation and SDS-PAGE. Native Hsp150Δ-β-lactamase was similarly treated in sec63-1 cells, at the restrictive temperature to block its translocation. These two proteins migrated similarly and slightly more slowly than the Hsp150Δ-β-lactamase variant lacking the signal peptide (II, Figure 3 B). Furthermore, E166A could not be labelled with mannose (II, Figure 3B). Taken together, the E166A fusion protein bound to PenG was associated with functional translocons on the ER membrane, but was not inserted far enough to be reached by the signal peptidase or O-glycosylation apparatus.

3.4. The effect of reversibly bound drug on translocation (II)

The experiments with the Hsp150Δ-β-lactamaseE166D mutant, which binds PenG in a reversible manner (Adachi et al., 1991), supported the view that the irreversible folding of the β-lactamase portion of the fusion protein resulted in its accumulation on the cytosolic face of the ER membrane. The translocation efficiency of the E166D mutant was found not to be affected by PenG (II, Figure 1 C and D). Neither was the translocation of native Hsp150Δ-β-lactamase affected by another antibiotic, Cloxacillin (Clx), which is hydrolyzed by the enzyme (Citri and Zytk, 1982). That reversible binding allowed translocation indicates that the ligand had to be displaced for translocation.

3.5. An unfolding step prior to translocation (I, II)

Since the release of the ligand allowed translocation across the ER membrane, we suggest that unfolding of pretranslocationally folded domains is required for successful translocation. To determine the putative unfolding step, Hsp150Δ-β-lactamase was expressed in three different strains deficient in translocation, namely sec61-41, sec63-1 and kar2-159. In the first, docking of secretory protein to the translocon occurs, but the precursor is not inserted on the channel at the restrictive temperature of 17°C (Pilon et al., 1998). The latter two are defective in posttranslational translocation. In kar2-159 cells, BiP is not functional at the restrictive temperature (Vogel et al., 1990), and in sec63-1, the J-domain of the protein is unable to stimulate the ATPase activity of BiP (Lyman and Schekman, 1997). At 37°C, translocation was rather slow in sec61-41 cells (II, Figure 5), which allowed the use of this same temperature for all of these strains. As revealed by immunoprecipitation and SDS-PAGE, in all strains the amounts of fusion protein accumulated on the cytosolic face of the ER membrane were similar (II, Figure 5A). This was also observed in Western blotting analysis of isolated microsomes. Thus, we could compare the activities of β-lactamase in these strains to gain information on their conformations.
The cytoplasmic activities of β-lactamase increased in sec63-1 and kar2-159 cells in a similar manner during incubation at 37°C. In contrast, the activity in sec61-41 cells remained at a low level throughout the experiment (II, Figure 5B). Since there was no difference in protein expression in these strains, most of the fusion protein in the sec61-41 cells, in which translocation channels were incapable of accommodating polypeptides, was apparently in a non-native conformation. The inactivity does not, however, tell us much about the conformational stage of the protein, as even a small change in its three-dimensional structure could lead to inactivation. To obtain further information, the $T_m$ values and protease-resistance of Hsp150Δ-β-lactamases in the same translocation-defective cells were determined. The $T_m$ value for protein in sec61-41 cells was lower than that in sec63-1 cells (Figure 5C) and for previously measured native Hsp150Δ-β-lactamase (Holkeri and Makarow, 1998). For the protease-resistance assay, these cells were metabolically labelled at the restrictive temperature, Hsp150Δ-β-lactamase was immunoprecipitated in non-denaturing conditions and then subjected to trypsin digestion. In sec63-1 cells, the β-lactamase portion of the fusion protein remained stable in the presence of trypsin (II, Figure 6A). In contrast, in sec61-41 cells, most of the fusion protein was degraded under the same conditions (II, Figure 6A). Thus, in sec61-41 cells, where the fusion protein interacted with the translocation channel but was not allowed to penetrate through the pore, it seemed to be unfolded to a trypsin-sensitive form. The translocation defect in sec61-41 cells was reversible, since during the chase in the presence of CHX at the permissive temperature, the fusion protein was secreted to the culture medium (II, Figure 7).

To study the possible unfolding machinery in the yeast cytoplasm, the 66 kDa cytoplasmic form and the 110 kDa ER form of the reporter protein were accumulated in sec62-101, sec63-201 and sec18-1 cells, and subjected to coimmunoprecipitation with anti-Hsp70 antibody. The ER form accumulating in sec18-1 cells at the restrictive temperature was used as a reference. The cytoplasmic reporter protein was found in association with cytosolic Hsp70 chaperones (I, Figure 8), whereas the 110 kDa form of the reporter was not, indicating the specificity of the interaction. Preimmunoserum precipitated neither the reporter protein nor Hsp70.

4. Protein folding in the ER (III)

4.1. The effect of DTT on secretion (III)

In the preceding section, the folding of preproteins in the cytoplasm was described. Now we move to the other side of the ER membrane to study protein folding. The reducing agent, DTT, has been shown to penetrate yeast cell walls and membranes and to inhibit disulfide formation in the ER in a reversible manner (Jämsä et al., 1994). Many reduced secretory proteins and the Hsp150Δ-β-lactamase fusion protein are known to remain in the ER in the presence of DTT (Jämsä et al., 1994). The requirement of BiP for translocation of proteins into the ER has made it difficult to get in vivo information about its role in protein folding. The DTT-dependent secretion block was taken advantage of in studying the requirement of BiP for conformational maturation of two reporter proteins,
Hsp150Δ-β-lactamase and Hsp150Δ-NGFR.<

First, the effect of DTT on secretion of Hsp150Δ-NGFR was established. In non-reducing SDS-PAGE, native and reduced molecules migrate differently due to conformational differences. Hsp150Δ-NGFR was expressed and metabolically labelled with [35S]-methionine/cysteine in sec18-1 cells at the restrictive temperature of 37°C, where secretory proteins accumulate in the ER and ER-derived vesicles (Kaiser and Schekman, 1990), in the presence or absence of DTT (III, Figure 3B). When the migration rates in non-reducing SDS-PAGE were compared after immunoprecipitation with anti-Hsp150Δ-NGFR antiserum, the DTT-reduced fusion protein appeared to migrate more slowly than the native fusion protein. The removal of DTT by washing and chasing with CHX in the absence of DTT at the restrictive temperature resulted in molecules having similar migration rates to native fusion proteins. Thus, disulfide bond formation, occurring normally at 37°C, was prevented in the presence of DTT, and the reduced molecules were reoxidized in the ER after removal of DTT.

To study whether the reoxidized Hsp150Δ-NGFR molecules could be secreted, a similar labelling experiment was done in wild type cells (III, Figure 3A). Without DTT, the fusion protein was secreted to the culture medium. The addition of DTT nearly totally inhibited the secretion of the fusion protein, as very little protein could be detected in the culture medium. When DTT was removed, the fusion protein was again secreted. DTT treatment reversibly blocked the secretion of the fusion protein. After removal of the DTT-imposed secretion block, the secreted Hsp150D-NGFR molecules migrated more slowly than the native molecules in SDS-PAGE. The reduced molecules apparently were more effectively N-glycosylated.

However, in wild-type cells the DTT-treated molecules were only weakly detectable, raising the possibility that they could have been heterogeneously N-glycosylated in the Golgi. To confirm that DTT-treatment prevented secretion from the ER, labelling was performed in wild-type cells in the presence of both DTT and tunicamycin (TM) to inhibit N-glycosylation (III, Figure 4). The fusion protein was now clearly visible and migrated similarly as the ER-retained form of the fusion protein in sec18-1 cells. The Golgi form of the fusion protein in sec7 cells at the restrictive temperature migrated more slowly and similarly to the mature, secreted form of the fusion protein. Since the fusion protein in wild type cells in the presence of DTT apparently lacked extended glycans, it was likely to reside in the pre-Golgi compartment. Its poor detection can be due to the reduced affinity of antibodies to DTT-treated molecules, which have an altered conformation.

Next, the kinetics of resumption of a secretion-competent conformation was studied for the two reporter proteins. Wild type cells expressing Hsp150Δ-NGFR were pulse-labelled with DTT and chased for different times after removal of DTT. Hsp150Δ-NGFR was secreted to the culture medium very rapidly, in less than five minutes (III, Figure 7A). The addition of TM had no effect on the rate of secretion. Hsp150Δ-β-lactamase, similarly expressed and labelled, was secreted in about 40 minutes after re-establishing of oxidative conditions in the ER (III, Figure 7C). It thus required a considerably longer time for folding into a secretion-competent conformation after reoxidation than the NGFR fusion protein.
4.2. The role of BiP in conformational maturation and translocation (III)

To study the chaperoning role of BiP, the Hsp150Δ-NGFR<sub>e</sub> reporter protein was expressed in temperature-sensitive kar2-159 cells, in which BiP is irreversibly inactivated at the restrictive temperature of 34°C (Vogel et al., 1990). The translocation block was circumvented by using DTT at permissive temperature to prevent disulfide formation and secretion during labelling with 35S-methionine/cysteine, but not translocation into the ER, and thereafter diluting DTT at the nonpermissive temperature to inactivate the function of BiP, followed by chase in the presence of CHX for different times. Reference cells were treated similarly at the permissive temperature. At both temperatures, the reporter protein could be found in the culture medium (III, Figure 5A). The addition of TM had no effect on secretion of the reporter protein (III, Figure 5B). In both cases, the kinetics of secretion seemed to be similar. Hsp150Δ-NGFR<sub>e</sub> thus acquired disulfides and folded into a secretion-competent conformation in the absence of functional BiP and N-glycosylation.

The same experiment was performed on Hsp150Δ-β-lactamase. Without functional BiP, secretion of reduced and reoxidized Hsp150Δ-β-lactamase was severely impaired (III, Figure 6A). The conformational maturation of a yeast vacuolar protein CPY has been previously reported to be similarly dependent on the function of BiP (Simons et al., 1995). In the very same kar2-159 cells, the maturation of CPY indeed required BiP, whereas the maturation of the Hsp150Δ-NGFR<sub>e</sub> did not (III, Figure 6B). Thus, both CPY and Hsp150Δ-β-lactamase needed help from BiP for conformational maturation after removal of DTT. Under the same conditions, Hsp150Δ-NGFR<sub>e</sub> was capable of undergoing conformational maturation without functional BiP.

Finally, BiP was confirmed to be required for translocation of both reporter proteins. The reporter protein Hsp150Δ-NGFR<sub>e</sub> was labelled and chased at the restrictive temperature and immunoprecipitated from the cell lysates and culture medium. Under these conditions, the fusion protein was found in cell lysates as a faster migrating band as compared with the ER and mature forms (III, Figure 8). The fusion protein was apparently on the cytoplasmic side of the ER membrane, as it could not be labelled with mannose. Thus, yeast BiP has two separate functions, assisting translocation and chaperoning.
DISCUSSION

1. Pretranslational protein folding

Our studies demonstrated for the first time that proteins can fold in the cytosol into a native-like conformation before translocation into the ER. This is contrary to the previous view of the mechanism of posttranslational translocation, where cytoplasmic Hsp70 chaperones were suggested to maintain the polypeptide in a translocation-competent or unfolded form prior to translocation (Chirico et al., 1988; Deshaies et al., 1988). The Hsp150Δ-β-lactamase fusion protein, destined for posttranslational translocation, was folded into a trypsin-resistant, active conformation in the yeast cytosol not only in translocation-defective mutants but also in wild-type cells. In the lumen of the ER, the reporter protein was correctly modified, and then secreted to the culture medium (Figure 4). Authentic β-lactamase is a tight globular protein (Jelsch et al., 1992). Most of authentic Hsp150 does not adopt any regular secondary structure, with only the C-terminal domain, which is not present in Hsp150Δ-carrier, consisting of β-sheets, as determined by CD and NMR spectroscopy (Jämä et al., 1995a). The structural differences between Hsp150 and the fusion protein likely explain the difference in translational retardation, which appeared to occur due to cytoplasmic events concerning the β-lactamase portion. The similarity of the Kₙ values for both forms of the fusion protein on both sides of the ER membrane to that of authentic E.coli β-lactamase indicated that they shared similar structures at their activity sites.

Although we are first to report folding prior to translocation into the ER, as far as we know, similar observations have been made in studies of mitochondrial translocation. Folding of artificial reporter proteins and authentic mitochondrial cytochrome b₅ prior to translocation into mitochondria have been reported (Glick et al., 1993; Langer and Neupert, 1994; Wachter et al., 1994). In addition, Nguyen et al. (1991) have observed the existence of a protease-resistant form of a yeast secretory protein prepro-α-factor on the cytoplasmic side of the ER membrane when translocation was inhibited. They suggested that resistance occurred as a result of aggregation or tight association with the ER membrane.

Disulfide bond formation of the β-lactamase portion in the ER is essential for acquisition of an active and secretion-competent conformation. The reducing agent DTT prevents proper folding, and the fusion protein is retained in the ER (Simonen et al., 1994). However, in the cytoplasm, the fusion protein folded into an active conformation even though the disulfide bond was not formed, reflecting the differences of these compartments as folding milieus. Authentic β-lactamase has been shown to be active in the E.coli cytosol, where disulfides are not formed (Plückthun and Knowles, 1987). Minsky et al. (1986) reported that after translocation across the bacterial membrane β-lactamase occurs first as a trypsin-sensitive, membrane-bound form, which is then converted to a trypsin-resistant and active form, suggesting that the protein may be unfolded and refolded after translocation. Similarly, the dependence of the active conformation in the ER on the disulfide bond suggests that Hsp150Δ-β-
lactamase unfolds at least to some extent prior to, during or after translocation into the ER and refolds in the ER lumen. In addition, since the cysteines forming the disulfide bond are in the interior of the β-lactamase molecule (Jelsch et al., 1992), their oxidation would require at least partial unfolding of the protein.

2. Unfolding precedes translocation

The crystal structure of authentic β-lactamase has a size of 32 x 37 x 53 Å (Jelsch et al., 1992). As the diameter of the translocation pore was reported to enlarge up to 60 Å when translocating a polypeptide (Hamman et al., 1997), and the same translocation channel was shown to accommodate glycosylated incorrectly folded proteins on their way back to the cytosol (Pilon et al., 1997), the question arose whether Hsp150Δ-β-lactamase could be translocated in a folded or loosely folded conformation. To answer this, two β-lactamase mutants were fused to the Hsp150Δ fragment. One of these, E166A, irreversibly binds the antibiotic penicillin G (Adachi et al., 1991) and forms a stable complex (Strynadka et al., 1992). The advantage of this mutant is its ability to lock the β-lactamase into a folded structure, which is incapable of unfolding due to the covalently bound antibiotic. The other mutant, E166D, binds the same antibiotic in a reversible manner (Adachi et al., 1991) and confers reversibility to the stabilization of the conformation.

To be active, proteins must fold into correct three-dimensional structures. However, unfolding of proteins is also essential for several cell processes, such as ER translocation. β-lactamase, locked into a folded form due to the bound PenG, could not be translocated into the ER in living yeast cells. The observation that the signal peptide was uncleaved and that not even the first few of the multiple glycosylation sites of Hsp150Δ were occupied (Suntio et al., 1999), suggests that the fusion protein did not penetrate into the translocation channel. This was unexpected, since the Hsp150Δ fragment has no structural constraints for translocation, as it occurs mostly as a random coil (Jämsä et al., 1995a). It appears as though the apparently cytoplasmic unfolding machinery, the translocating substrate and the translocation machinery, communicate with each other, and that the translocation pore remains closed until completion of unfolding. PenG and another β-lactam antibiotic cloxacillin, which bind reversibly to E166D and native β-lactamase, respectively, did not prevent translocation of their respective fusion proteins. This suggests that the release of the drug allowed unfolding and subsequent translocation, and that prefolded proteins have to unfold for translocation. Similar results have been obtained in posttranslational translocation into the mitochondria both in vitro (Eilers and Schatz, 1986) and in vivo (Wienhues et al., 1991). Translocation of a fusion protein consisting of a mitochondrial targeting signal and mouse DHFR into isolated yeast mitochondria was prevented if DHFR was bound to its ligand, methotrexate. Methotrexate was found to stabilize the structure of DHFR, and thus was suggested to block translocation through mitochondrial membranes by preventing unfolding of the protein (Eilers and Schatz, 1986). Wienhues et al. (1991) reported a similar translocation block in vivo. By using a ligand of DHFR, which could be removed from the protein, they showed that the translocation block was reversible, further
supporting the necessity of unfolding for translocation. The import of folded preproteins into mitochondria can be hundreds of times faster than their spontaneous unfolding, indicating that mitochondria can actively unfold proteins (Matouschek et al., 1997).

The Hsp70 protein of the ER lumen, BiP, is important in driving posttranslational translocation of preproteins into the ER (Vogel et al., 1990). Hsp150Δ-β-lactamase was active on the cytoplasmic side of the ER membrane in the absence of functional BiP or its J-domain partner Sec63p. Translocation in these mutants was blocked at a stage which precedes the unfolding step. According to the results obtained with sec61-41 cells, where the insertion of preproteins into the translocation channel is prevented at the restrictive temperature, Hsp150Δ-β-lactamase appeared to be unfolded to an inactive and trypsin-sensitive conformation when docked to the translocation channel, while the entire fusion protein was still on the cytoplasmic side of the ER membrane (Figure 4). In other words, the fusion protein was unfolded in the cytoplasm while bound to translocation channels, and before it had been reached by lumenal signal peptidase complex and BiP. In a reconstituted system with purified translocation components in the absence of a lipid bilayer, efficient translocation of prepro-α-factor was achieved even if BiP was not present but substituted by antibodies against the translocating polypeptide (Matlack et al., 1999). However, folding of prepro-α-factor was not studied, and it might not achieve a folded structure prior to translocation. Our results demonstrate that a prefolded protein is unfolded in vivo while the entire polypeptide resides on the cytoplasmic side of the ER membrane. Thus, BiP does not actively pull and thereby unfold the translocating polypeptide. After unfolding and translocation into the ER, Hsp150Δ-β-lactamase was refolded in the ER and secreted to the medium.

Despite translocation of proteins into the ER and mitochondria sharing common features, the mechanisms of unfolding seem to be different. The length of the presequence of mitochondrial reporter proteins has been shown to affect the import rate. Unfolding of a preprotein at the

![Figure 4. Model for cytoplasmic folding and unfolding of Hsp150D-b-lactamase. The b-lactamase portion of the fusion protein folds to a native-like conformation in the yeast cytosol prior to translocation. Disulfide bond is not formed. When attached to the Sec61 complex, b-lactamase unfolds to inactive and protease sensitive form even though the whole fusion protein still resides on the cytosolic side of the ER membrane. After translocation into the ER, the signal peptide is removed, the Hsp150D is O-glycosylated, the disulfide bond is formed, and the protein refolds to a conformation with similar catalytic properties as the cytosolic form.](image-url)
mitochondrial surface was accelerated when its presequence was sufficiently long to span both mitochondrial membranes and to interact with Hsp70 of the mitochondrial matrix (Matouschek et al., 1997). The unfolding pathway of a mitochondrial barnase preprotein was distinct from its spontaneous unfolding pathway (Huang et al., 1999). Just like many enzymes catalyze chemical reactions by changing reaction pathways, the protein import machinery catalyzes protein unfolding by changing the unfolding pathway of the substrate protein. Temperature-sensitive mutants of mitochondrial matrix Hsp70, which lead to its inactivation, inhibit unfolding, translocation and subsequent folding of mitochondrial precursors inside the mitochondria (Gambill et al., 1993; Voos et al., 1993). Mitochondrial Hsp70s are the major consumers of ATP during translocation (Glick et al., 1993). They seem to function as active motors together with Tim44, a mitochondrial inner membrane protein, by pulling the precursor across the membrane (Voisine et al., 1999). The N-terminal amino acids of an F_0–ATPase subunit were translocated, and only the fusion partner, DHFR, stabilized by methotrexate, was exposed to the cytoplasmic side of the mitochondrial membrane (Voisine et al., 1999). Import of loosely folded precursors have been reported to occur in the absence of functional Tim44 (Bömer et al., 1998; Merlin et al., 1999), but a stably folded preprotein must be actively pulled and thus unfolded, and not only trapped, by matrix Hsp70s (Bömer et al., 1998; Merlin et al., 1999; Voisine et al., 1999). Thus, in mitochondria, matrix Hsp70 unfolds preproteins for translocation.

As Hsp150Δβ-lactamase was almost entirely exposed to the cytosol during unfolding, the unfolding machinery must be cytosolic. The cytoplasmic reporter protein interacted with cytoplasmic Hsp70 chaperones as shown by coimmunoprecipitation experiments, and the ER form interacted with the lumenal Hsp70, BiP (Jämsä et al., 1995). In spite of the high homology of these proteins, they cannot substitute for each other in translocation across the ER membrane (Brodsky et al., 1993). Hsp150Δβ-lactamase existed in a folded conformation in the cytoplasm prior to its translocation, even though it was in association with cytoplasmic Hsp70 proteins. This could perhaps indicate a role for Ssa proteins in unfolding of pretranslocationally folded secretory proteins. Translocation of pre-pro-α-factor and folding of nascent luciferase have been demonstrated in vitro that to be independent of the functional Ssa1 and Ssa2 proteins, whereas the refolding of denatured luciferase requires the presence of these chaperones (Bush and Meyer, 1996). Ssa proteins were thus proposed to unfold the translocation-incompetent structures in the yeast cytosol before translocation. β-lactamase must unfold for translocation, but to what extent it unfolds remains to be studied. The folding pathway of most proteins in vitro contains a partially folded intermediate known as the molten globule. Proteins have been postulated to be in the molten globule state during translocation across membranes (Bychkova et al., 1988). By introducing disulfide bridges that covalently linked two to five β-sheets together, Schwartz et al. (1999) demonstrated in vitro that preproteins are normally fully unfolded during translocation into mitochondria, but residual structures can be imported, although less efficiently. The diameter of the protein conducting channel of the outer mitochondrial membrane reconstituted in lipid vesicles was measured to be 20 Å (Künkele et al., 1998), thus sufficient to accommodate
secondary structural elements. It is not yet known whether the channel enlarges during translocation. Nor can it be ruled out that mitochondrial membranes might contain a range of import channels of different sizes.

3. The functions of BiP in translocation and folding are distinct

Since unfolding of pretranslocationally folded proteins precedes translocation, the proteins must refold in the ER lumen. The involvement of BiP/Kar2p in translocation as well as in facilitating folding complicates in vivo studies on its role in folding. The use of DTT to prevent folding of translocated proteins helped to define the two functions of BiP, and made it possible to study its role in folding in vivo. The two reporter proteins used in this study, E. coli β-lactamase and the ectodomain of rat nerve growth factor receptor (NGFR), have different three-dimensional structures, which likely explains their different requirements of BiP in conformational maturation after reducing conditions. DTT treatment led to the retention of both reporter proteins in the ER. This has also been shown to occur in mammalian cells, where reduced VSV G protein was retained in the ER, as revealed by cell fractionation and morphological studies (Hammond and Helenius, 1994), and in yeast, as DTT-reduced CPY lacked extended glycan structures, indicating its localization in the pre-Golgi compartment (Holst et al., 1996). When retained under reduced conditions in the ER, the NGFR_e portion of the fusion protein was more efficiently N-glycosylated than in normal conditions, where the N-glycosylation site is variably occupied (Holkeri et al., 1996; Simonen et al., 1996). Thus, folding and glycosylation seem to compete with each other, and folding of polypeptides prior to glycosylation hides the potential glycosylation site, thereby preventing glycosylation. The reducing conditions keep the N-glycosylation site of NGFR_e available for the oligosaccharyltransferase over a prolonged period, leading to an increased level of modification.

Hsp150Δ-NGFR_e folded to an apparently correct three-dimensional structure and was allowed to leave the ER to be secreted in conditions where BiP was not functioning and could not facilitate folding. As a multidomain protein, NGFR_e probably folds domain by domain when emerging from the translocation channel (Netzer and Hartl, 1997). This decreases the enormous number (3.16 x 10^11) of possible disulfide bonds formed by 24 cysteines of NGFR_e to 15. In human TNF receptor (TNFR), the cysteines seem to form disulfides in sequence with the first possible free cysteine. Furthermore, in the case of TNFR, the cysteine residues were not critical to folding (Banner et al., 1993). This indicates that perhaps the folding of NGFR_e is not severely complicated in reduced conditions; the protein is able to acquire a nearly correct conformation even in the absence of disulfides, which would quickly be formed if oxidizing conditions were resumed in the ER. This would explain why the protein is able to mature to a secretion-competent conformation so rapidly.

As a globular protein, β-lactamase probably needs to be fully translocated into the ER before the final structure can be attained. The disulfide bond resides in the middle of the molecule and its formation presumably requires that the surrounding areas are kept unfolded or loosely folded. BiP binds to unfolded nascent polypeptide sequences (Simons et al., 1995; Hendershot et al.,...
This could explain the requirement of BiP for folding of β-lactamase. BiP could be coimmunoprecipitated with Hsp150Δβ-lactamase (Jämsä et al., 1995b), whereas no interaction between Hsp150Δ-NGFR and BiP was detected (data not shown). In kar2-159 cells, the ATPase activity of BiP is defective (Vogel et al., 1990), leading to stable binding of BiP to its substrates (Palleros et al., 1993). Mutant BiP was likely to be bound permanently to reduced β-lactamase, thereby preventing folding, supporting the view that BiP would favour folding by preventing aggregation.

Taken together, we have shown that facilitation of translocation and folding are two separate functions of BiP. Furthermore, although BiP is required for posttranslational translocation of all secretory proteins studied thus far, its function in folding is substrate-specific. Proteins that easily fold into native conformations perhaps do not need to be aided by BiP. Differences in requirements of BiP for posttranslational chaperoning have been reported in mammalian cells (Pittman et al., 1994; Morris et al., 1997). Recently, Hellman et al. (1999) showed that the rate and stability of protein folding determines whether or not a particular site is recognized by BiP. BiP was found to bind preferentially to slowly folding or unstable proteins.

CONCLUSIONS

The results of this study show for the first time that in living S. cerevisiae cells posttranslationally translocated proteins can fold into native-like conformations on the cytosolic face of the ER membrane. When attached to the translocon, prefolded proteins unfold for translocation. The unfolding step occurs before initiation of penetration through the translocation pore, thus revealing a novel function in the yeast cytosol, that of unfolding of prefolded proteins. In the ER, translocated proteins refold into secretion-competent conformation. Even though chaperone BiP is required for translocation of all protein substrates, its requirement for conformational maturation was found to be protein-specific.
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[Signature]
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