Protein Folding before and after Translocation into the Yeast Endoplasmic Reticulum

Eija Paunola

Institute of Biotechnology,
Department of Biosciences, Division of Biochemistry,
and
Graduate School in Microbiology and
Viikki Graduate School in Biosciences,
Faculty of Science
University of Helsinki
Finland

Academic dissertation

To be presented, with the permission of the Faculty of Science of the University of Helsinki, for public criticism in the auditorium 1041 at Viikki Biocenter (Viikinkaari 5, Helsinki) on August 11th, 2000, at 12 o'clock.

Helsinki 2000

Supervised by

Professor Marja Makarow
Institute of Biotechnology
University of Helsinki

Reviewed by

Docent Pekka Lappalainen
Institute of Biotechnology
University of Helsinki

Doctor Vesa Kontinen
Department of Vaccines
National Public Health Institute
Helsinki

Opponent

Docent Kalervo Metsikkö
Department of Anatomy
University of Oulu

Kustos

Professor Carl G. Gahmberg
Department of Biosciences
Division of Biochemistry
University of Helsinki

ISBN 951-45-9454-1 (nid)
ISBN 952-91-2284-5 (verkkojulkaisu, pdf)
ISSN 1239-9469

Yliopistopaino
Helsinki 2000

ORIGINAL PUBLICATIONS

This thesis is based on the following articles and a manuscript, which are referred to in the text by their Roman numerals.

I Paunola E., Suntio T., Jämsä E. and Makarow M. 1998. Folding of active β-lactamase in the yeast cytoplasm before translocation into the endoplasmic reticulum. Mol. Biol. Cell 9: 817-827.

II Paunola E., Qiao M. and Makarow M. Prefolded β-lactamase is unfolded *in vivo* before translocation into the yeast ER. (submitted)

III Holkeri H., Paunola E., Jämsä E. and Makarow M. 1998. Dissection of the translocation and chaperoning functions of yeast BiP/Kar2p *in vivo*. J. Cell Sci. 111: 749-757.

CONTENTS

SUMMARY **1**

INTRODUCTION **2**

1. Overview **2**

2. Protein translocation **3**

2.1. Protein targeting to the endoplasmic reticulum **3**
2.1.1. Signal peptides 3
2.1.2. Cotranslational translocation 3
2.1.3. Posttranslational translocation 5
2.2. The translocation channel **5**
2.3. Chaperones in translocation **9**
2.4. Retrotranslocation **10**

3. Protein maturation in the ER **11**

3.1. Glycosylation **12**
3.1.1. N-glycosylation 12
3.1.2. O-glycosylation 12
3.2. Lectin-like chaperones: Calnexin and calreticulin **13**
3.3. Classical chaperones **14**
3.4. Folding enzymes: Protein disulfide isomerases and peptidyl prolyl isomerases **15**

AIMS OF THE STUDY **17**

MATERIALS AND METHODS **18**

RESULTS **21**

1. The reporter proteins: Hsp150 and the fusion proteins **21**

2. Protein folding before translocation into the ER (I) **22**

2.1. Hsp150D is translocated posttranslationally (I) **22**
2.2. The translocation intermediate on the cytoplasmic face of the ER membrane (I) **22**
2.3. The translocation intermediate is active (I) **23**

3. The conformation of proteins attached to the translocon (II) **23**

3.1. The b-lactamase mutants (II) **23**
3.2. The effect of PenG on the reporter (II) **24**
3.3. Subcellular localization of PenG-bound reporter protein (II) **24**
3.4. The effect of reversibly bound drug on translocation (II) **25**
3.5. An unfolding step prior to translocation (I, II) **25**

4. Protein folding in the ER (III) **26**

4.1. The effect of DTT on secretion (III) **26**
4.2. The role of BiP in conformational maturation and translocation (III) **28**

DISCUSSION **29**

1. Pretranslocational protein folding **29**

2. Unfolding precedes translocation **30**

3. The functions of BiP in translocation and folding are distinct **33**

CONCLUSIONS **34**

ACKNOWLEDGEMENTS **35**

REFERENCES **36**

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
$NGFR_e$	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 ($NGFR_e$) 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 $NGFR_e$ 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-$NGFR_e$ were used to study if these two functions were independent. Translocation of Hsp150Δ-β-lactamase and Hsp150Δ-$NGFR_e$ 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 $NGFR_e$, 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.

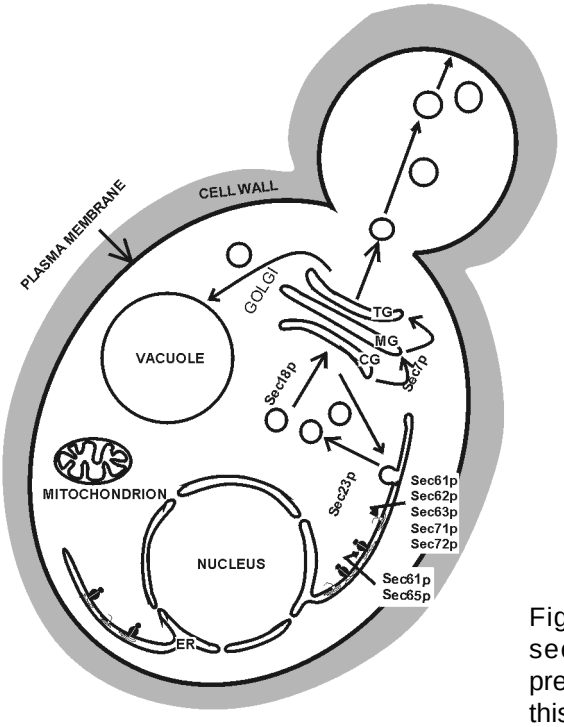


Figure 1. A schematic picture of the secretory pathway of *S. cerevisiae* presenting the Sec proteins mentioned in this study.

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 (Paetzel *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* showes 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; Regnacq *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 (Deshaies 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

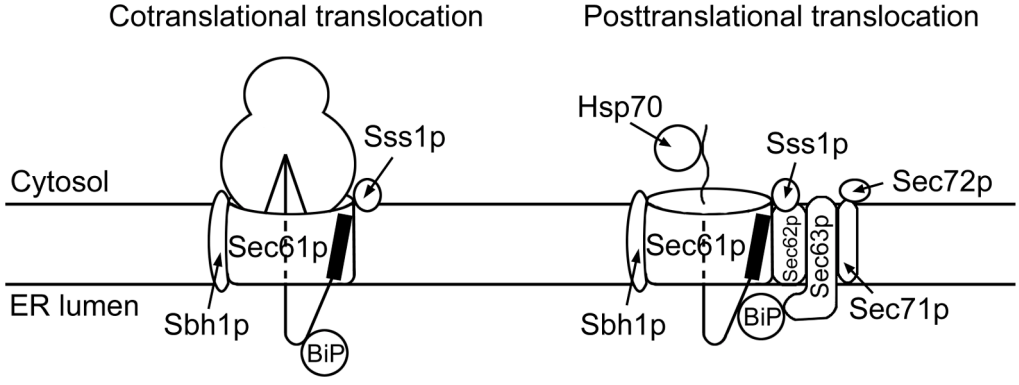


Figure 2. Co- and posttranslational protein translocation in yeast. In cotranslational translocation the Sec61 complex mediates protein import into the ER. Ribosome binds tightly to the channel thereby sealing it from the cytoplasmic side. In posttranslational translocation the Sec61 complex associates 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 lumenal 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 fluorecent 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 lumenal 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 (Deshaies *et al.* , 1991; Panzner *et al*., 1995) (Figure 2). The complete heptameric complex is required for posttranslational translocacation 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 (Deshaies *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 lumenal 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 *SSA1* 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 lumenal 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 translocase. Thus, even in cotranslational translocation, completion of translocation may be dependent on chaperones. Indeed, Nicchitta and Blobel (1993) provided evidence that lumenal components are required for net transfer of secretory proteins to mammalian microsomes. Depletion of the lumenal 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 lumenal proteins, indicating that lumenal 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), illustratng 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 lumenal side must be triggered by a mechanism that is different from that used during translocation into the ER.

A mutant form of carboxy peptidase 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 retro-translocation 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 (Abeijon 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 coimmuno-precipitation 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 $Glc_3Man_9GlcNac_2$ 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 oligo-saccharyltransferase 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 oligo-saccharides (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 (Spiro *et al*., 1996). A calnexin homologue, the product of *CNE1* gene in yeast, is not essential for viability (Parlati *et al*., 1995). It may, however, function in quality control similar to its mammalian counterparts. For example, in Δ*cne1* cells, a temperature-sensitive mutant protein that is retained in the ER in wild type cells, was secreted (Parlati *et al*., 1995).

The monoglucosylated N-linked oligosaccharides arise as transient intermediates during the processing of N-glycans in the ER (Ware *et al*., 1995; Spiro *et al*., 1996). These structures are generated either by the action of glucosidases I and II or by glucosyl-transferase re-adding a glucose residue to the trimmed glycans (Helenius *et al*., 1997). Helenius *et al*. (1997) proposed a quality control function for calnexin and calreticulin. UDP-glucose:glycoprotein glucosyltransferase (UGGT) was suggested to be a folding sensor: if the conformation of the glycoprotein was not native, it would re-add the glucose residue. This enables the incompletely folded protein to rebind to calnexin or calreticulin. 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 fo 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 *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 thioredixin 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 *PDI1,* 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 gluthathione 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 gluthathione 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.

Strain number	Relevant genotype	Reporter protein	Publication	Source or reference
H1	wt		I	Schekman R
H4	*sec18-1*		I, II	Kaiser and Schekman, 1990
H22	*kar2-159*		II	Rose *et al.*, 1989
H257	*sec61-3*		II	Schekman R
H272	*sec65-1*		I	Stirling *et al.*, 1992
H259	*sec63-1*		I, II	Schekman R
H335	wt	Hsp150Δ-β-lactamase	I, II, III	Simonen *et al.*, 1994
H393	*sec18-1*	Hsp150Δ-β-lactamase	I, II	Simonen *et al.*, 1994
H401	*Δerg6*		II	Jackson and Képès, 1994
H426	*wt, Δhsp150*	Hsp150Δ-NGFRe	III	Simonen *et al.*, 1996
H430	*sec7-1*	Hsp150Δ-β-lactamase	III	Simonen *et al.*, 1996
H482	*sec63-1*	Hsp150Δ-β-lactamase	I, II	This study
H495	*kar2-159*	Hsp150Δ-β-lactamase	II, III	This study
H501	*sec61-3*	Hsp150Δ-β-lactamase	II, III	Jämsä *et al.*, 1995
H538	*sec18-1 Δhsp150*	Hsp150Δ-NGFRe	III	Holkeri *et al.*, 1996
H613	*kar2-159 Δhsp150*	Hsp150Δ-NGFRe	III	This study
H664	*sec65-1*	Hsp150Δ-β-lactamase	I	This study
H681	*sec62-101*		I	Ng *et al.*, 1996
H682	*sec63-201*		I	Ng *et al.*, 1996
H694	*sec62-101*	Hsp150Δ-β-lactamase	I	This study
H695	*sec63-201*	Hsp150Δ-β-lactamase	I	This study
H977	wt	Δ1-20Hsp150Δ-β-lactamase	II	This study
H987	*Δerg6*	Hsp150Δ-β-lactamase	II	This study
H1016	*sec61::HIS3* p*SEC61*		II	Pilon *et al.*, 1997
H1018	*sec61::HIS3* p*sec61-41*		II	Pilon *et al.*, 1997
H1030	*sec61::HIS3* p*SEC61*	Hsp150Δ-β-lactamase	II	This study
H1032	*sec61::HIS3* p*sec61-41*	Hsp150Δ-β-lactamase	II	This study
H1045	*Δerg6*	Hsp150Δ-β-lactamaseE166A	II	This study
H1046	*Δerg6*	Hsp150Δ-β-lactamaseE166D	II	This study

Table 2. Relevant defects of the mutant yeast strains.

Mutant	Defect	Reference
sec61-3	ER translocation	Stirling *et al.*, 1992
sec61-41	ER translocation	Pilon *et al.*, 1997
sec62-101	Posttranslational ER translocation	Ng *et al.*, 1996
sec63-201	Posttranslational ER translocation	Ng *et al.*, 1996
sec63-1	Posttranslational ER translocation	Rothblatt *et al.*, 1989
sec65-1	Cotranslational ER translocation	Stirling *et al.*, 1992
sec18-1	Vesicle fusion from ER to Golgi	Kaiser and Schekman, 1990
sec7-1	Trafficing in Golgi	Franszusoff and Schekman, 1989
kar2-159	BiP's ATPase	Vogel *et al.*, 1990
Δerg6	Sterol composition of membranes	Jackson and Képès, 1994

Table 3. The methods used in this study.

Method	Publication
Plasmid construction	I, II
Yeast transformation	I, II, III
β-lactamase assay	I, II
Immunofluorescense	II
Isolation of microsomes	I
Metabolic labeling with (^{35}S) methionine/cysteine	I, II, III
Metabolic labeling with (^{3}H) mannose	I, II
Immunoprecipitation	I, II, III
Coimmunoprecipitation	I
Trypsin digestion	I, II
SDS PAGE and non-reducing SDS-PAGE	I, II, III
Western analysis	I, II

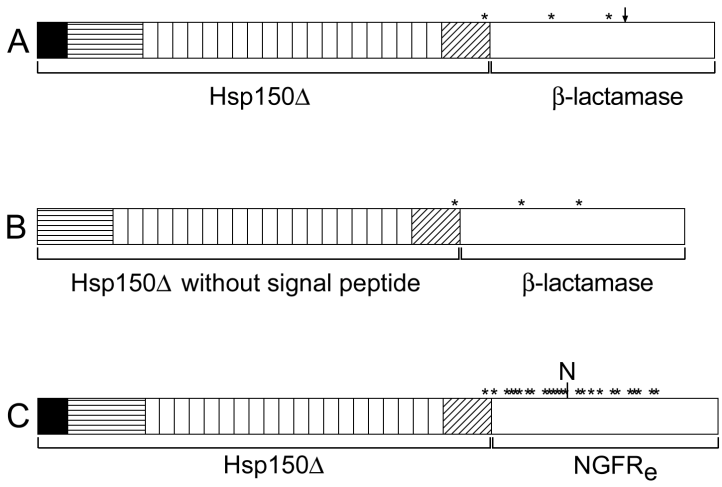


Figure 3. Schematic presentation of the reporter proteins used in this study. A) Hsp150Δ-β-lactamase, B) Δ1-18Hsp150Δ-β-lactamase and C) Hsp150Δ-NGFR$_e$. The Hsp150Δ consists of subunit I (horizontally striped) and an N-terminal part of the subunit II composed of a repetetive region (11 white boxes) 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 NGFR$_e$ 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.

RESULTS

1. The reporter proteins: Hsp150 and the fusion proteins

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) ($NGFR_e$), 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 ($TNFR_e$) (Radeke *et al.*, 1987) and the crystal structure of $TNFR_e$ (Banner *et al.*, 1993), $NGFR_e$ is likely to have an extended rod-like structure of four domains, each of which have three intradomain disulfide bonds (III, Figure 2). $NGFR_e$ 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 $NGFR_e$ were anticipated to challenge the folding apparatus in yeast ER.

2. Protein folding before translocation into the ER (I)

2.1. Hsp150Δ 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 Hsp150Δ-β-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 Hsp150Δ-β-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, Hsp150Δ-β-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 Hsp150Δ-β-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-β-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 K_m values for nitrocefin of cytoplasmic and ER forms of Hsp150Δ-β-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 Hsp150Δ-β-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 [^{35}S]-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 3 A). 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 immuno-precipitation 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 in the presence of PenG (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 Zyk, 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 coimmuno-precipitation 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-$NGFR_e$.

First, the effect of DTT on secretion of Hsp150Δ-$NGFR_e$ was established. In non-reducing SDS-PAGE, native and reduced molecules migrate differently due to conformational differences. Hsp150Δ-$NGFR_e$ was expressed and metabolically labelled with [^{35}S]-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-$NGFR_e$ 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-$NGFR_e$ 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-$NGFR_e$ 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 heterogenously 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 temparature 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-$NGFR_e$ were pulse-labelled with DTT and chased for different times after removal of DTT. Hsp150Δ-$NGFR_e$ 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 $NGFR_e$ fusion protein.

4.2. The role of BiP in conformational maturation and translocation (III)

To study the chaperoning role of BiP, the Hsp150Δ-NGFR$_e$ 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 ^{35}S-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$_e$ 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$_e$ 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$_e$ 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$_e$ 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. Pretranslocational 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 (Jämsä *et al*., 1995a). The structural differences between Hsp150 and the fusion protein likely explain the difference in translocational retardation, which appeared to occur due to cytoplasmic events concerning the β-lactamase portion. The similarity of the K_m 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 b_2 prior to translocation into mitochondria have been reported (Glick *et al*., 1993; Langer and Neupert, 1994; Wachter *et al*., 1994). In addition, Nguen *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 (Eiliers 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 membane (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 mitochodrial reporter proteins has been shown to affect the import rate. Unfolding of a preprotein at the

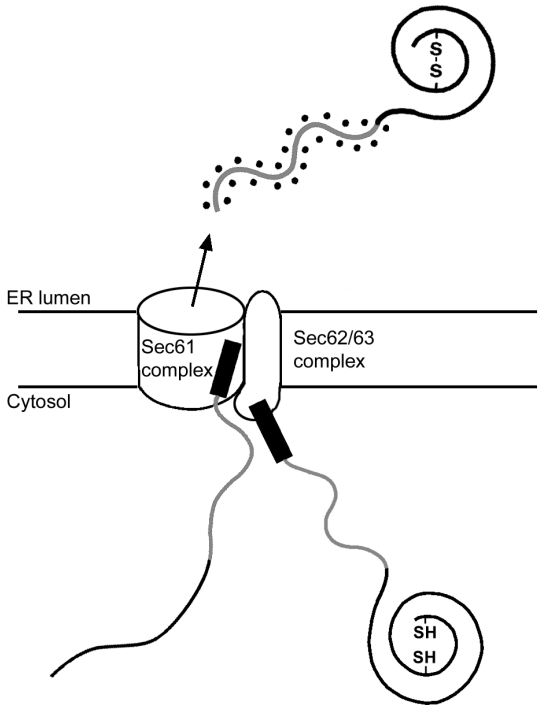


Figure 4. Model for cytoplasmic folding and unfolding of Hsp150D-b-lactamase. The b-lactamase portion of the fusion protein folds to a native-like conformation in the yeast cytosol prior to translocation. Disulfide bond is not formed. When attached to the Sec61 complex, b-lactamase unfolds to inactive 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.

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 Hsp150Δ-β-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 coimmuno-precipitation 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). Hsp150Δ-β-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 ($NGFR_e$), 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 (3.16×10^{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*.,

1996).This could explain the requirement of BiP for folding of β-lactamase. BiP could be coimmunoprecipitated with Hsp150Δ-β-lactamase (Jämsä *et al*., 1995b), whereas no interaction between Hsp150Δ-$NGFR_e$ 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 posttranslocational 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.

ACKNOWLEDGEMENTS

This work was carried out at the Institute of Biotechnology, with the financial support of the Graduate School in Microbiology and the Viikki Graduate School in Biosciences.

I wish to express my gratitute to my supervisor, Professor Marja Makarow, for the oportunity to work in her group. Her enthusiasm, positive attitude and continuous interest made working in the yeast group a pleasure.

I thank Professor Mart Saarma, the head of the Institute of Biotechnology, for providing excellent working facilities and creating a dynamic, stimulating and warm working environment. Professor Leevi Kääriäinen I thank for the detailed timetable regarding my dissertation. I thank also professor Timo Korhonen, the head of the Graduate School in Microbiology. I am grateful to Docent Pekka Lappalainen and Dr. Vesa Kontinen for their critical reading of the manuscript. I sincerely thank them for pointing out the sections of the text which were probably written after falling into a deep sleep. Heli Ruoho is acknowledged for the layout.

I warmly thank all the former and present members of the yeast group for the lively, lovely and loud atmosphere at work. Our tea breaks, all day long with a good laugh, made brighter even the gloomiest moments. I will never forget the most enjoyable parties both at work and in private, they really shook me all night long!

I especially wish to thank Marjo Simonen and Eija Jokitalo for the very nice welcome to the yeast group and the advice in the beginning, and Eija for her friendship ever since and the criticism without mercy not only at work but also at shopping. I thank Anna Liisa Nyfors for the excellent technical help, patience and her good eyes in finding lost bottles and tubes. To Heidi, Nina, Mari, Netta, Eeva, Taina, Ansku, Maria, Laura and Leena I owe my thanks for friendship, helpful hands in every day problems and shaping activities outside of work. The boys, Vijay, Anton, Juha and Qiao, in spite of trying hard to not to be different than the rest of us, reaching a climax when they dressed up as women, have provided a welcome masculinity to the traditionally quite feminine appearance of the yeast group. I also thank Qiao for the efficient cloning work in the last article.

I thank my parents and relatives for their love and support even though I never became a lawyer. All my friends are thanked for the relaxing time together. I owe my special thanks to Hanna for the long-distance calls whenever I needed a shrink! My deepest thanks go to Olli and Topi, for their love and trust and more precisely, Olli for creating order and Topi for creating refreshing disorder in the study. Topi, your training of my skills in debate with a determination of a three-year old is acknowledged!
Helsinki, August 2000



REFERENCES

Abeijon C. and Hirschberg C. 1992. Topography of glycosylation reactions in the endoplasmic reticulum. TIBS 17: 32-36.

Adachi H., Ohta T. and Matsuzava H. 1991. Site-directed mutants, at position 166, of RTEM-1 β-lactamase that form a stable acyl-enzyme intermediate with penicillin. J. Biol. Chem. 266: 3186-3191.

Arnold C. and Wttrup K. 1994. The stress response to loss of signal recognition particle function in Saccharomyces cerevisiae. J. Biol. Chem. 269: 30412-30418.

Arunachalam B. and Cresswell P. 1995. Molecular requirements for the interaction of class II major histocompatibility complex molecules and invariant chain with calnexin. J. Biol. Chem. 270: 2784-2790.

Bacher G., Lütcke H., Jungnickel B., Rapoport T. and Dobberstein B. 1996. Regulation by the ribosome of the GTPase of the signal-recognition particle during protein targeting. Nature 381: 248-251.

Banner D. W., D'Arcy A., Janes W., Gentz R., Schenfeld H-J., Broger C., Loetscher H. and Lesslauer W. 1993. Crystal structure of the soluble human 55 kd TNF receptor-Human TNFb complex: Implications for TNF receptor activation. Cell 73: 431-445.

Baxter B., James P, Evans T. and Craig E. A. 1996. SSI1 encodes a novel Hsp70 of the *Saccharomyces cerevisiae* endoplasmic reticulum. Mol. Cel. Biol. 16: 6444-6456.

Beckman R., Bubeck D., Grassucci R., Penczek P., Verschoor A., Blobel G. and Frank J. 1997. Alignment of conduits for the nascent polypeptide chain in the ribosome-Sec61 complex. Science 278: 2123-2126.

Bergeron J., Brenner M., Thomas D. and Williams D. 1994. Calnexin: A membrane-bound chaperone of the endoplasmic reticulum. TIBS 19: 124-128.

Biederer T., Volkwein C. and Sommer T. 1996. Degradation of subunits of the Sec61p complex, an integral component of the ER membrane, by the ubiquitin-proteasome pathway. EMBO J. 15: 2069-2076.

Blobel G. and Dobbestein B. 1975. Transfer of proteins across membranes I. presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on the membrane-bound ribosomes of murine myeloma. J. Cell Biol. 67: 835-851.

Blond-Elguindi S., Cwirla S., Dower W., Lipshutz S., Sprang S., Sambrook J. and Gething M-J. 1993. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. Cell 75: 717-728.

Bordallo J., Plemper R. K., Finger A. and Wolf D. H. 1998. Der3p/Hrd1p is required for the endoplasmic reticulum degradation of misfolded lumenal and integral membrane proteins. Moll. Biol. Cell 9: 209-222.

Brodsky J. and Schekman R. 1993. A Sec63p-BiP complex from yeast is required for protein translocation in a reconstituted proteoliposome. J. Cell Biol. 123: 1355-1363.

Brodsky J. L., Werner E. D., Dubas M. E., Goeckeler J. L., Kruse K. B. and McCracken A. A. 1999. The requirement for molecular chaperones during endoplasmic reticulum-associated protein degradation demonstrates that protein export and import are mechanistically distinct. J. Biol. Chem. 274: 3453-3460.

Brodsky J., Goeckeler J. and Schekman R. 1995. BiP and Sec63p are required for both co- and posttranslational translocation into the yeast endoplasmic reticulum. Proc. Natl. Acad. Sci. USA 92: 9643-9646.

Brodsky J., Hamamoto S., Feldheim D. and Schekman R. 1993. Reconstitution of protein translocation from solubilized yeast membranes reveals topolocally distinct roles for BiP and cytosolic Hsc70. J. Cell Biol. 120: 95-102.

Brown J., Hann B., Medzihradszky K., Niwa M., Burlingame A. and Walter P. 1994. Subunits of the *Saccharomyces cerevisiae* signal recognition particle required for its functional expression. EMBO J. 13: 4390-4400.

Bukau B. and Horwich A. 1998. The Hsp70 and Hsp60 chaperone machines. Cell 92: 351-366.

Bush G. and Meyer D. 1996. The refolding activity of the yeast heat shock proteins Ssa1 and Ssa2 defines their role in protein translocation. J. Cell Biol. 135: 1229-1237.

Bychkova V., Pain R. and Ptitsyn O. 1988. The molten globule state is involved in the translocation of proteins across the membranes? FEBS Lett. 238: 231-234.

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

Bömer U., Maarse A. C., Martin F., Geissler A., Merlin A., Schönfisch B., Meijer M., Pfanner N. and Rassow J. 1998. Separation of structural and dynamic functions of the mitochondrial translocase: Tim44 is crusial for the inner membrane import sites in translocation of tightly folded domains, but not of loosely folded preproteins. EMBO J. 17: 4226-4237.

Cannon K. and Helenius A. 1999. Trimming and readdition of glucose to N-linked oligosaccharides determines calnexin association of a substrate glycoprotein in living cells. J. Biol. Chem. 274: 7537-7544.

Cannon K., Hebert D. and Helenius A. 1996. Glycan-dependent and –independent association of vesicular stomatitis virus G protein with calnexin. J. Biol. Chem. 271: 14280-14284.

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

Caplan A., Cyr D. and Douglas M. 1992. YDJ1p facilitates polypeptide translocation across different intracellular membranes by a conserved mechanism. Cell 71: 1143-1155.

Chevet E., Wong H., Gerber D., Cochet C., Fazel A., Cameron P., Gushue J., Thomas D. and Bergeron J. 1999. Phosphorylation by CK2 and MAPK enhances calnexin assosiation with ribosomes. EMBO J. 18: 3655-3666.

Chirico W., Waters M. and Blobel G. 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature 332: 805-810.

Citri Nand Zyk N. 1982. Interaction of the pBR 322-coded RTEM b-lactamase with substrates. Biochem. J. 201: 425-427.

Connolly T. and Gilmore R. 1989. The signal recognition particle receptor mediates the GTP-dependent displacement of SRP from the signal sequence of the nascent polypeptide. Cell 57: 599-610.

Connolly T., Rapiejko P. and Gilmore R. 1991. Requirement of GTP hydrolysis for dissociation of the signal recognition particle from its receptor. Science 252: 1171-1173.

Corsi A. and Schekman R. 1996. Mechanism of polypeptide translocation into the endoplasmic reticulum. J. Biol. Chem. 271: 30299-30302.

Corsi A. and Schekman R. 1997. The lumenal domain of Sec63p stimulates the ATPase activity of BiP and mediates BiP recruitment to the translocon in *Saccharomyces cerevisiae*. J. Cell Biol. 137: 1483-1493.

Craven R., Egerton M. and Stirling C. 1996. A novel Hsp70 of the yeast ER lumen is required for the efficient translocation of a number of protein precursors. EMBO J. 15: 2640-2650.

Crowley K., Liao S., Worrell V., Reinhart G. and Johnson A. 1994. Secretory proteins move through the endoplasmic reticulum membrane via an aqueos, gated pore. Cell 78: 461-471.

Crowley K., Reinhart G. and Johnson A. 1993. The signal sequence moves through a ribosomal tunnel into a noncytoplasmic aqueous environment at the ER membrane early in translocation. Cell 73: 1101-1115.

Cyr D., Lu X. and Douglas M. 1992. Regulation of Hsp70 function by a eukaryotic DnaJ homolog. J. Biol. Chem. 267: 20927-20931.

Darby N., Penka E. and Vincentelli R. 1998. The multi-domain structure of protein disulfide isomerase is essential for high catalytic efficiency. J. Mol. Biol. 276, 239-247.

Deshaies R. and Schekman R. 1987. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. J. Cell Biol. 105, 633-645.

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

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

Dünnwald M., Varshavsky A. and Johnsson N. 1999. Detection of transient *in vivo* interactions between substrate and transporter during protein translocation into the endoplasmic reticulum. Mol. Biol. Cell 10: 329-344.

Edman J., Ellis L., Blacher R., Roth R. and Rutter W. 1985. Sequence of protein disulfide isomerase and implications of its relationship to thioredoxin. Nature 317: 267-270.

Eilers M. and Schatz G. 1986. Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. Nature 322: 228-232.

Esnault Y., Blondel M., Deshaies R., Sheckman R. and Képès F. 1993. The yeast *SSS1* gene is essential for secretory protein translocation and encodes a conserved protein of the endoplasmic reticulum. EMBO J. 12: 4083-4093.

Fang H., Mullins C. and Green N. 1997. In addition to *SEC11*, a newly identified gene, *SPC3*, is essential for signal peptidase activity in the yeast endoplasmic reticulum. J. Biol. Chem. 272: 13152-13158.

Feldheim D. and Schekman R. 1994. Sec72p contributes to the selective recognition of signal peptides by the secretory polypeptide translocation complex. J. Cell Biol. 126: 935-943.

Finke K., Plath K., Panzner S., Prehn S., Rapoport T., Hartmann E. and Sommer T. 1996. A second trimeric complex containing homologs of the Sec61p complex functions in protein transport across the ER membrane of *S. cerevisiae*. EMBO J. 15: 1482-1494.

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

Frand A. and Kaiser C. 1998. The *ERO1* gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. Mol. Cell 1: 161-170.

Frand A. and Kaiser C. 1999. Ero1p oxidases protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. Mol. Cell 4, 469-477.

Franzusoff A. and Schekman R. 1989. Functional compartments of the yeast Golgi apparatus are defined by the *sec7* mutation. EMBO J. 8: 2695-2702.

Freedman R. 1984. Native disulphide bond formation in protein biosynthesis: Evidence for the role of protein disulfide isomerase. TIBS, 438-441.

Freedman R., Hirst T. and Tuite M. 1994. Protein disulfide isomerase: Building bridges in protein folding. TIBS 19: 331-336.

Gambill B. D., Voos W., Kang P. J., Miao B., Langer T., Craig E. A. and Pfanner N. 1993. A dual role for mitochondrial heat shock protein 70 in membrane translocation of preproteins. J. Cell Biol. 123: 109-117.

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

Gillece P., Luz J., Lennartz W., de la Cruz F. and Römisch K. 1999. Export of a cysteine-free misfolded secretory protein from the endoplasmic reticulum for degradation requires interaction with protein disulfide isomerase. J. Cell Biol. 147: 1443-1456.

Gilmore R., Blobel G. and Walter P. 1982. Protein translocation across the endoplasmic reticulum. I. Detection in the microsomal membrane of a receptor for the signal recognition particle. J. Cell Biol. 95: 463-469.

Glick B. 1995. Can Hsp70 proteins act as force-generating motors? Cell 80: 11-14.

Glick B. S., Wachter C., Reid G. A. and Schatz G. 1993. Import of cytochrome b_2 to the mitochondrial intermembrane space: The tightly folded heme-binding domain makes import dependent upon matrix ATP. Protein Science 2: 1901-1917.

Graham K., Le A. and Sifers R. 1990. Acumulation of the insoluble PiZ variant of human a1-antitrypsin within the hepatic endoplasmic reticulum does not elevate the steady-state level of grp78/BiP. J. Biol. Chem. 265: 20463-20468.

Green N, Fang H. and Walter P. 1992. Mutants in three novel complementation groups inhibit membrane protein insertion into and soluble protein translocation across the endoplasmic reticulum membrane of *Saccharomyces cerevisiae*. J. Cell Biol. 116, 597-604.

Gruss O., Feick P., Frank R. and Dobberstein B. 1999. Phosphorylation of components of the ER translocation site. Eur. J. Biochem. 260: 785-793.

Görlich D., Hartmann E., Prehn S. and Rapoport T. 1992a. A protein of the endoplasmic reticulum involved early in polypeptide translocation. Nature 357: 47-52.

Görlich D., Prehn S., Hartmann E., Kalies K-U. and Rapoport T. 1992b. A mammalian homolog of SEC61p and SECYp is associated with ribosomes and nascent polypeptides during translocation. Cell 71: 489-504.

Haas I. and Wabl M. 1983. Immunoglobulin heavy chain binding protein. Nature 306: 387-389.

Hamilton T. and Flynn G. 1996. Cer1p, a novel Hsp70-related protein required for the posttranslational endoplasmic reticulum translocation in yeast. J. Biol. Chem. 271: 30610-30613.

Hamilton T., Norris T., Tsuruda P. and Flynn G. 1999. Cer1p functions as a molecular chaperone in the endoplasmic reticulum of *Saccharomyces cerevisiae*. Mol. Cel. Biol. 19: 5298-5307.

Hamman B., Chen J-C., Johnson E. and Johnson A. 1997. The aqueous pore through the translocon has a diameter of 40-60 Å during cotranslational protein translocation at the ER membrane. Cell 89: 535-544.

Hamman B., Hendershot L. and Johnson A. 1998. BiP maintains the permeability barrier of the ER membrane by sealing the lumenal end of the translocon pore before and early in translocation. Cell 92: 747-758.

Hammond C. and Helenius A. 1994. Quality control in the secretory pathway. Retention of a misfolded viral membrane glycoprotein involves cycling between the ER, intermediate

compartment, and the Golgi apparatus. J. Cell Biol. 126: 41-52.

Hammond C., Braakman I. and Helenius A. 1994. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. Proc. Natl. Acad. Sci. USA 91: 913-917.

Hampton R. Y., Gardner R. G. and Rine J. 1996. Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. Mol. Biol. Cell 7: 2029-2044.

Hanein D., Matlack K., Jungnickel B., Plath K., Kalies K-U., Miller K., Rapoport T. and Akey C. 1996. Oligomeric rings of the Sec61p complex induced by ligands required for protein translocation. Cell 87: 721-732.

Hann B. and Walter P. 1991. The signal recognition particle in *S. cerevisiae*. Cell 67: 131-144.

Hann B., Stirling C. and Walter P. 1992. *SEC65* gene product is a subunit of the yeast signal recognition particle required for its integrity. Nature 356: 532-533.

Hartmann E., Sommer T., Prehn S., Görlich D., Jentsch S. and Rapoport T. 1994. Evolutionary conservation of components of the protein translocation complex. Nature 367: 654-657.

Haselbeck A. and Tanner W. 1983. O-glycosylation in *Saccharomyces cerevisiae* is initiated at the endoplasmic reticulum. FEBS Lett. 158: 335-338.

Hedge R., Voigt S., Rapoport T. and Lingappa V. 1998. TRAM regulates the exposure of nascent secretory proteins to the cytosol during translocation into the endoplasmic reticulum. Cell 92: 621-631.

Helenius A., Trombetta S., Hebert D. and Simons J. 1997. Calnexin, calreticulin and the folding of glycoproteins. Trends Cell Biol. 7: 193-200.

Hellman R., Vanhove M., Lejeune A., Stevens F. and Hendershot L. 1999. The *in vivo* association of BiP with newly synthesized proteins is dependent on the rate and stability of folding and not simply on the presence of sequences that can bind to BiP. J. Cell Biol. 144: 21-30.

Hendershot L., Wei J., Gaut J., Melnick J., Aviel S. and Argon Y. 1996. Inhibition of immunoglobulin folding and secretion by dominant negative BiP ATPase mutants. Proc. Natl. Acad. Sci. USA 93: 5269-5274.

High S. and Dobberstein B. 1991. The signal sequence interacts with the methionine-rich domain of the 54-kD protein of signal recognition particle. J. Cell Biol. 113: 229-233.

High S. and Dobberstein B. 1992. Mechanism that determine the transmembrane disposition of proteins. Curr. Opinion Cell Biol. 4: 581-586.

High S., Martoglio B., Görlich D., Andersen S., Ashford A., Giner A., Hartmann E., Prehn S., Rapoport T., Dobberstein B. and Brunner J. 1993. Site-specific photocross-linking reveals that Sec61p and TRAM contact different regions of a membrane-inserted signal sequence. J. Biol. Chem. 268: 26745-26751.

Hiller M. M., Finger A., Schweiger M. and Wolf D. H. 1996. ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. Science 273: 1725-1728.

Holkeri H. and Makarow M. 1998. Different degradation pathways for heterologous glycoproteins in yeast. FEBS Lett. 429: 162-166.

Holkeri H., Simonen M., Pummi T., Vihinen H. and Makarow M. 1996. Glycosylation of rat NGF receptor ectodomain in the yeast *Saccharomyces cerevisiae.* FEBS Lett. 383: 255-258.

Holst B., Bruun A. W., Kielland-Brandt M. C. and Winther J. R. 1996. Competition between folding and glycosylation in the endoplasmic reticulum. EMBO J. 15: 3538-3546.

Huang S., Ratliff K., Schwartz M., Spenner J. and Matouschek A. 1999. Mitochondria unfold precursor proteins by unraveling them from their N-termini. Nature Struc. Biol. 6: 1132-1138.

Hwang C., Sinskey A. Lodish H. 1992. Oxidized redox state of glutathione in the endoplasmic reticulum. Science 257: 1496-1502.

Immervoll T., Gentzsch M. and Tanner W. *PTM3* and *PTM4*, two new members of the protein-O-mannosyltransferase gene family of *Saccharomyces cerevisiae*. Yeast 11: 1345-1351.

Jackson C. and Képès F. 1994. *BFR1*, a multicopy suppressor of Brefeldin A induced lethality, is implicated in secretion and nuclear segregation in *Saccharomyces cerevisiae*. Genetics 137: 423-437.

Jakob C., Burda P., te Heesen S., Aebi M. and Roth J. 1998. Genetic tailoring of N-linked oligosaccharides: The role of glucose residues in glycoprotein processing of *Saccharomyces cerevisiae in vivo*. Glycobiology 8: 155-164.

Jelsch C., Lenfant F., Masson J. M. and Samana J.P. 1992. β-Lactamase TEM1 of *E.coli*. Crystal structure determination at 2.5 Å resolution. FEBS Lett. 299: 135-142.

Jungnickel B. and Rapoport T. 1995. A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane. Cell 82: 261-270.

Jämsä E., Holkeri H., Vihinen H., Wikström M., Simonen M., Walse B., Kalkkinen N., Paakkola J. and Makarow M. 1995a. Structural features of a polypeptide carrier promoting secretion of a β-lactamase fusion protein in yeast. Yeast 11: 1381-1391.

Jämsä E., Simonen M. and Makarow M. 1994. Selective retention of secretory proteins in the yeast endoplasmic reticulum by treatment of cells with a reducing agent. Yeast 10: 335-370.

Jämsä E., Vakula N., Arffman A., Kilpeläinen I. and Makarow M. 1995b. *In vivo* reactivation of heat-denatured protein in the endoplasmic reticulum of yeast. EMBO J. 14: 6028-6033.

Kaiser C. A. and Schekman R. 1990. Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. Cell 61, 723-733.

Kalies K-U., Görlich D. and Rapoport T. 1994. Binding of ribosomes to the rough endoplasmic reticulum mediated by the Sec61p-complex. J. Cell Biol. 126: 925-934.

Kalies K-U., Rapoport T. and Hartmann E. 1998. The b subunit of the Sec61 complex facilitates cotranslational protein transport and interacts with the signal peptidase during translocation. J. Cell Biology 141, 887-894.

Kassenbrock C. and Kelly R. 1989. Interaction of heavy chain binding protein (BiP/GRP78) with adenine nucleotides. EMBO J. 8: 1461-1467.

Keenan R., Freymann D., Walter P. and Stroud R. 1998. Crystal structure of the signal sequence binding subunit of the signal recognition protein. Cell 94: 181-191.

Kelleher D., Kreibich G. and Gilmore R. 1992. Oligosaccharyltransferase activity is associated with a protein complex composed of ribophorins I and II and a 48 kd protein. Cell 69: 55-65.

Kemmink J., Darby N., Dijkstra K., Nilges M. and Creighton T. 1997. The folding catalyst protein disulfide isomerase is constructed of active and inactive thioredoxin modules. Current Biology 7: 239-245.

Kiefhaber T., Quaas R., Hahn U. and Schmid F. 1990. Folding of ribonuclease T_1. Kinetic models for the folding and unfolding reactions. Biochem. 29: 3061-3070.

Kim P. and Arvan P. 1995. Calnexin and BiP act as sequential molecular chaperones during thyroglobulin folding in the endoplasmic reticulum. J. Cell Biol. 128: 29-38.

Klappa P., Ruddock L., Darby N. and Freedman R. 1998. The b' domain provides the principal

peptide-binding site of protein disulfide isomerase but all domains contribute to binding of misfolded proteins. EMBO J. 17: 927-935.

Knop M., Finger A., Braun T., Hellmuth K. and Wolf D. H. 1996. Der1, a novel potein specifically required for endoplasmic reticulum degradation in yeast. EMBO J. 15: 753-763.

Kohno K., Normington K., Sambrook J., Gething M-J. and Mori K. 1993. The promoter region of the yeast *KAR2* gene contains a regulatory domain that responds to the presence of unfolded proteins in the endoplasmic reticulum. Mol. Cel. Biol. 13: 877-890.

Krieg U., Walter P. and Johnson A. 1986. Photocrosslinking of the signal sequence of nascent preprolactin to the 54-kilodalton polypeptide of the signal recognition particle. Proc. Natl. Acad. Sci. USA 83: 8604-8608.

Kukuruzinska M., Bergh M. and Jackson B. 1987. Protein glycosylation in yeast. Ann. Rev. Biochem. 56: 915-944.

Kurzhalia T., Wiedmann M., Girshovich A., Bochkareva E., Bielka H. and Rapoport T. 1986. The signal sequence of nascent preprolactin interacts with the 54K polypeptide of the signal recognition particle. Nature 320: 634-636.

Künkele K-P., Heins S., Dembowski M., Nargang F., Benz R., Thieffry M., Walz J., Lill R., Nussberger S. and Neupert W. 1998. The preprotein translocation channel of the outer membrane of mitochondria. Cell 93: 1009-1019.

Laboissière M., Sturley S. and Raines R. 1995. The essential function of protein disulfide isomerase is to unscramble non-native disulfide bonds. J. Biol. Chem. 270: 28006-28009.

Langer T. and Neupert W. 1994. Chaperoning mitochondrial biogenesis. In: The biology of heat shock proteins and molecular chaperones, ed. R. Morimoto, A. Tissiers, and C. Georgopoulos, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 53-83.

Lehle L. and Bause E. 1984. Primary structural requirements for N- and O-glycosylation of yeast mannoproteins. Biochim. Biophys. Acta 799: 246-251.

Lodish H., Kong N. and Wikström L. 1992. Calsium is required for folding of a newly made subunits of the asialoglycoprotein receptory within the endoplasmic reticulum. J. Biol. Chem. 267: 12753-12760.

Lussier M., Gentzsch M., Sdicu A-M., Bussey H. and Tanner W. 1995. Protein O-glycosylation in yeast. J. Biol. Chem. 270: 2770-2775.

Lyman S. and Schekman R. 1995. Interaction between BiP and Sec63p is required for the completion of protein translocation into the ER of *Saccharomyces cerevisiae*. J. Cell Biol. 131: 1163-1171.

Lyman S. and Schekman R. 1997. Binding of secretory precursor polypeptides to a translocon subcomplex is regulated by BiP. Cell 88: 85-96.

Martoglio B., Hofmann M., Brunner J. and Dobberstein B. 1995. The protein-conducting channel in the membrane of the endoplasmic reticulum is open laterally toward the lipid bilayer. Cell 81: 207-214.

Matlack K., Misselwitz B., Plath K. and Rapoport T. 1999. BiP acts as a molecular ratchet during posttranslational transport of prepro-a-factor across the ER membrane. Cell 97: 553-564.

Matlack K., Mothes W. and Rapoport T. 1998. Protein translocation: Tunnel vision. Cell 92: 381-390.

Matlack K., Plath K., Misselwitz B. and Rapoport T. 1997. Protein transport by purified yeast Sec complex and Kar2p without membranes. Science 277: 938-941.

Matoba S. and Ogrydziak D. 1998. Another factor besides hydrophobicity can affect signal peptide interaction with signal recognition particle. J. Biol. Chem. 273: 18841-18847.

Matouschek A., Azem A., Ratliff K., Glick B., Schmid K. and Schatz G. 1997. Active unfolding of precursor proteins during mitochondrial protein import. EMBO J. 16: 7627-6736.

McClellan A., Endres J., Vogel J., Palazzi D., Rose M. and Brodsky J. 1998. Specific molecular chaperone interactions and an ATP-dependent conformational change are required during posttranslational protein translocation into the yeast ER. Mol. Biol. Cell 9: 3533-3545.

Meaden P., Hill K., Wagner J., Slipetz D., Sommer S. and Bussey H. 1990. The yeast *KRE5* gene encodes a probable endoplasmic reticulum protein required for (1→6)-β-D-glucan synthesis and normal cell growth. Mol. Cel. Biol. 10: 3013-3019.

Melnick J., Dul J. and Argon Y. 1994. Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. Nature 370: 373-375.

Merlin A., Voos W., Maarse A., Meijer M., Pfanner N. and Rassow J. 1999. The J-related segment of Tim44 is essential for cell viability: A mutant Tim44 remains in the mitochondrial import site, but inefficiently recruits mtHsp70 and impairs protein translocation. J. Cell Biol. 145: 961-972.

Meyer D., Krause E. and Dobberstein B. 1982. Secretory protein translocation across membranes – the role of the 'docking protein'. Nature 297, 647-650.

Meyer H. and Hartmann E. 1997. The yeast SPC22/23 homolog Spc3p is essential for signal peptidase activity. J. Biol. Chem. 272: 13159-13164.

Minsky A., Summers R. G. and Knowles J. R. 1986. Secretion of b-lactamase into periplasm of *Escherichia coli*: Evidence for a distinct release step associated with a conformational change. Proc. Natl. Acad. Sci. USA 83: 4180-4184.

Misselwitz B., Staeck O. and Rapoport T. 1998. J proteins catalytically activate Hsp70 molecules to trap a wide range of peptide sequences. Mol. Cell 2: 593-603.

Misselwitz B., Staeck O., Matlack K. and Rapoport T. 1999. Interaction of BiP with the J-domain of the Sec63p component of the endoplasmic reticulum protein translocation complex. J. Biol. Chem. 274: 20110-20115.

Mori K., Sant A., Kohno K., Normington K., Gething M-J. and Sambrook J. 1992. A 22 bp *cis*-acting element is necessary and sufficient for the induction of the yeast *KAR2* gene by unfolded proteins. EMBO J. 11: 2583-2593.

Morris J., Dorner A., Edwards C., Hendershot L. and Kaufman R. 1997. Immunoglobulin binding protein (BiP) function is required to protect cells from endoplasmic reticulum stress but is not required for the secretion of selective proteins. J. Biol. Chem. 272: 4327-4334.

Mothes W., Jungnickel B., Brunner J. and Rapoport T. 1998. Signal sequence recognition in cotranslational translocation by components of the endoplasmic reticulum membrane. J. Cell Biol. 142: 355-364.

Mothes W., Prehn S. and Rapoport T. 1994. Systematic probing of the environment of a translocating secretory protein during translocation through the ER membrane. EMBO J. 13: 3973-3982.

Müsch A., Wiedmann M. and Rapoport T. 1992. Yeast Sec proteins interact with polypeptides traversing the endoplasmic reticulum membrane. Cell 69: 343-352.

Möller I., Jung M., Beatrix B., Levy R., Kreibich G., Zimmermann R., Wiedmann M. and Lauring B. 1998. A general mechanism for regulation of access to the translocon: Competition for a membrane attachment site on ribosomes. Proc. Natl. Acad. Sci. USA 95: 13425-13430.

Netzer W. and Hartl F. U. 1997. Recombination of protein domains facilitated by co-translational folding in eukaryotes. Nature 388: 343-349.

Neuhof A., Rolls M., Jungnickel B., Kalies K-U. and Rapoport T. 1998. Binding of signal

recognition particle gives ribosome/nascent chain complexes a competitive advantage in the endoplasmic reticulum membrane interaction. Mol. Biol. Cell 9: 103-115.

Ng D., Brown J. and Walter P. 1996. Signal sequences specify the targeting route to the endoplasmic reticulum membrane. J. Cell Biol. 134: 269-278.

Nguyen T., Law D. and Williams D. 1991. Binding protein BiP is required for translocation of secretory proteins into the endoplasmic reticulum in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 88: 1565-1569.

Nicchitta C. and Blobel G. 1993. Lumenal proteins of the mammalian endoplasmic reticulum are required to complete protein translocation. Cell 73, 989-998.

Normington K., Kohno K., Kozutsumi Y., Gething M-J. and Sambrook J. 1989. *S.cerevisiae* encodes an essential protein homologous in sequence and function to mammlian BiP. Cell 57: 1223-1236.

Ogg S., Poritz M. and Walter P. 1992. Signal recognition particle receptor is important for cell growth and protein secretion in *Saccharomyces cerevisiae*. Mol. Biol. Cell 3: 895-911.

Oliver J., van der wal F., Bulleid N. and High S. 1997. Interaction of the thiol-dependent reductase Erp57 with nascent glycoproteins. Science 275: 86-88.

Ou W-J., Cameron P., Thomas D. and Bergeron J. 1993. Association of folding intermediates of glycoproteins with calnexin during protein maturation. Nature 364: 771-776.

Paetzel M., Dalbey R. and Strynadka N. 1998. Crystal structure of a bacterial signal peptidase in complex with a b-lactam inhibitor. Nature 396: 186-190.

Palleros D., Reid K., Shi L., Welch W. and Fink A. 1993. ATP-induced protein-Hsp70 complex dissociation requires K^+ but not ATP hydrolysis. Nature 365: 664-666.

Panzner S., Dreier L., Hartmann E., Kostka S. and Rapoport T. 1995. Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. Cell 81: 561-570.

Parlati F., Domingues M., Bergeron J. and Thomas D. 1995. *Saccharomyces cerevisiae CNE1* encodes an endoplasmic reticulum (ER) membrane protein with sequence similarity to calnexin and calreticulin and functions as a constituent of the ER quality control apparatus. J. Biol. Chem. 270: 244-253.

Partaledis J. and Berlin V. 1993. The *FKB2* gene of S*accharomyces cerevisiae*, encoding the immunosuppressant-binding protein FKBP-13, is regulated in response to accumulation of unfolded proteins in the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA 90: 5450-5454.

Pilon M., Römisch K., Quach D. and Schekman R. 1998. Sec61p serves multiple roles in secretory precursor binding and translocation into the endoplasmic reticulum membrane. Mol. Biol. Cell 9: 3455-3473.

Pilon M., Schekman R. and Römisch K. 1997. Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. EMBO J. 16: 4540-4548.

Pittman D.D., Tomkinson K. N. and Kaufman R.J. 1994. Post-translational requirements for functional Factor V and Factor VIII secretion in mammalian cells. J. Biol. Chem. 269:17329-17337.

Plath K., Mothes W., Wilkinson B., Stirling C. and Rapoport T. 1998. Signal sequence recognition in posttranslational protein transport across the yeast ER membrane. Cell 94: 795-807.

Plemper R. K., Bordallo J., Deak P. M., Taxis C., Hitt R. and Wolf D. H. 1999b. Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retrotranslocation complex mediating protein transport for ER degradation. J. Cell Sci. 112: 4123-4134.

Plemper R. K., Böhmler S., Bordallo J., Sommer T. and Wolf D. H. 1997. Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. Nature 388: 891-895.

Plemper R. K., Deak P. M., Otto R. T. and Wolf D. H. 1999a. Re-entering the translocon from the lumenal side of the endoplasmic reticulum. Studies on mutated carboxypeptidase yscY species. FEBS Lett. 443: 241-245.

Plückthun A. and Knowles J. R. 1987. The consequences of stepwise deletions from the signal-processing site of β-lactamase. J. Biol. Chem. 262:3951-3957.

Pollard M., Travers K. and Weissman J. 1998. Ero1p: A novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. Mol. Cell 1: 171-182.

Radeke M. J., Misko T. P., Hsu C., Herzenberg L. A. and Shooter E.M. 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325: 593-597.

Rapiejko P. and Gilmore R. 1997. Empty site forms of the SRP54 and SRa GTPases mediate targeting of ribosome-nascent chain complexes to the endoplasmic reticulum. Cell 89, 703-713.

Regnacq M., Hewitt E., Allen J., Rosamund J. and Stirling C. 1998. Deletion analysis of yeast Sec65p reveals a central domain that is sufficient for function *in vivo*. Mol. Microbiol. 29: 753-762.

Rose M., Misra L. and Vogel J. 1989. *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. Cell 57: 1211-1221.

Rothblatt J. A., Deshaies R. J., Sanders S. L., Daum G. and Schekman R. 1989. Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. J. Cell Biol. 109: 2641-2652.

Rudd K., Sofia H., Koonin E., Plunket G., Lazar S. and Rouviere P. 1995. A new family of peptidyl-prolyl isomerases. Trends Biochem Sci 20: 12-14.

Russo P., Kalkkinen N., Sareneva H., Paakkola J. and Makarow M. 1992. A heat shock gene from *Saccharomyces cerevisiae* encoding a secretory glycoprotein. Proc. Natl. Acad. Sci. USA 89: 3671-3675.

Sambrook J. F. 1990. The involvement of calsium in transport of secretory proteins from the endoplasmic reticulum. Cell 61: 197-199.

Sanders S., Whitfield K., Vogel J., Rose M. and Schekman R. 1992. Sec61p and BiP directly facilitate polypeptide translocation into the ER. Cell 69: 353-365.

Saris N., Holkeri H., Craven R., Stirling C. and Makarow M. 1997. The Hsp70 homologue Lhs1p is involved in a novel function of the yeast endoplasmic reticulum, refolding and stabilization of heat-denatured protein aggregates. J. Cell Biol. 137: 813-824.

Saris N. and Makarow M. 1998. Transient ER retention as stress response: conformational repair of heat-damaged proteins to secretion-competent structures. J. Cell Sci. 111: 1575-1582.

Scherens B., Dubois E. and Messenguy F. 1991. Determination of the sequence of the yeast *YCL313* gene localized on the chromosome III. Homology with the protein disulfide isomerase (PDI gene product) of other organism. Yeast 7: 185-193.

Schlenstedt G., Gudmundsson G., Boman H. and Zimmermann R. 1990. A large presecretory protein translocates both cotranslationally, using signal recognition particle and ribosome, and posttranslationally, without these ribonucleoparticles, when synthesized in the presence of mammalian microsomes. J. Biol. Chem. 265: 13960-13968.

Schröder K., Martoglio B., Hofmann M., Hölscher C., Hartman E., Prehn S., Rapoport T. and Dobberstein B. 1999. Control of glycosylation of MHC class II-associated invariant chain by translocon-associated RAMP4. EMBO J. 18: 4804-4815.

Schwartz M., Huang S. and Matouschek A. 1999. The structure of precursor proteins during import into mitochondria. J. Biol. Chem. 274: 12759-12764.

Scidmore M., Okamura H. and Rose M. 1993. Genetic interactions between *KAR2* and *SEC63*, encoding eukaryotic homologues of DnaK and DnaJ in the endoplasmic reticulum. Mol. Biol. Cell 4: 1145.1159.

Shamu C., Cox J. and Walter P. 1994. The unfolded-protein-response pathway in yeast. Trends Cell Biol. 4: 56-60.

Shaw A., Rottier P. and Rose J. 1988. Evidence for the loop model of signal-sequence insertion into the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA 85: 7592-7596.

Siegel V. and Walter P. 1985. Elongation arrest is not a prerequisite for secretory protein translocation across the microsomal membrane. J. Cell Biol. 100: 1913-1921.

Siegel V. and Walter P. 1988. Each of the activities of signal recognition particle is contained within a distinct domain: Analysis of biochemical mutants of SRP. Cell 52: 39-49.

Simon S. and Blobel G. 1991. A protein-conducting channel in the endoplasmic reticulum. Cell 65, 371-380.

Simon S. and Blobel G. 1992. Signal pepides open protein-conducting channels in *E. coli.* Cell 69: 677-648.

Simon S., Peskin C. and Oster G. 1992. What drives the translocation of proteins? Proc. Natl. Acad. Sci. USA 89: 3770-3774.

Simonen M., Jämsä E. and Makarow M. 1994. The role of the carrier protein and disulfide formation in the folding of β-lactamase fusion proteins in the endoplasmic reticulum of yeast. J. Biol. Chem. 269: 13887-13892.

Simonen M., Vihinen H., Jämsä E., Arumäe U., Kalkkinen N. and Makarow M. 1996. The hsp150Δ-carrier confers secretion competence to the rat nerve growth factor receptor ectodomain in *Saccharomyces cerevisiae*. Yeast 12: 457-466.

Simons J., Ferro-Novick S., Rose M. and Helenius A. 1995. BiP/Kar2p serves as a molecular chaperone during carboxypeptidase Y folding in yeast. J. Cell Biol. 130: 41-49.

Skowronek M., Rotter M. and Haas I. 1999. Molecular characterization of a novel mammalian DnaJ-like Sec63p homolog. Biol. Chem. 380: 1133-1138.

Song W., Raden D., Mandon E. and Gilmore R. 2000. Role of Sec61α in the regulated transfer of the ribosome-nascent chain complex from the signal recognition particle to the translocon. Cell 100: 333-343.

Spee P., Subjeck J. and Neefes J. 1999. Identification of novel peptide binding proteins in the endoplasmic reticulum: ERp72, calnexin and grp170. Biochemistry 38: 10559-10566.

Spiro R., Zhu Q., Bhoyroo V. and Söling H-D. 1996. Definition of the lectin-like properties of the molecular chaperone, calreticulin, and demonstration of its copurification with endomannosidase from rat liver Golgi. J. Biol. Chem. 271: 11588-11594.

Stirling C. and Hewitt E. 1992. The *S.cerevisiae SEC65* gene encodes a component of yeast signal recognition particle with homology to human SRP19. Nature 356: 534-537.

Stirling C., Rothblatt J., Hosobuchi M., Deshaies R. and Schekman R. 1992. Protein translocation mutants defective in the insertion of integral membrane proteins into the endoplasmic reticulum. Mol. Biol. Cell 3: 129-142.

Strynadka N., Adachi H., Jensen S., Johns K., Sielecki A., Betzel C., Sutoh K. and James M.

1992. Molecular structure of the acyl-enzyme intermediate in β-lactam hydrolysis at 1.7 Å resolution. Nature 359: 700-105.

Suntio T., Shmelev A., Lund M. and Makarow M. 1999. The sorting determinant quiding Hsp150 to the COPI-independent transport pathway in yeast. J. Cell Sci. 112, 3889-3898.

Suzuki C., Bonifacino J., Lin A., Davis M. and Klausner R. 1991. Regulating the retention of T-cell receptor a chain variants within the endoplasmic reticulum: Ca^{2+}-dependent association with BiP. J. Cell Biol. 114: 189-205.

Tanner W. and Lehle L. 1987. Protein glycosylation in yeast. Biochim. Biophys. Acta 906: 81-99.

Tatu U., Hammond C. and Helenius A. 1995. Folding and oligomerization of influenza hemagglutinin in the ER and the intermediate compartment. EMBO J 14: 1340-1348.

Toikkanen J., Gatti E., Takei K., Saloheimo M., Olkkonen V., Söderlund H., de Camilli P. and Keränen S. 1996. Yeast protein translocation complex: Isolation of two genes *SEB1* and *SEB2* encoding proteins homologuos to the Sec61b subunit. Yeast 12: 425-438.

Trombetta E. and Helenius A. 1998. Lectins as chaperones in glycoprotein folding. Curr. Opinion in Structural Biol. 8: 587-592.

Wada I., Rindress D., Cameron P., Ou W-J., Doherty J., Louvard D., Bell A., Dignard D., Thomas D. and Bergeron J. 1991. SRa and associated calnexin are major calsium binding proteins of the endoplasmic reticulum membrane. J. Biol. Chem. 266: 19599-19610.

Walter P. and Blobel G. 1981. Translocation of proteins across the endoplasmic reticulum III. Signal recognition protein (SRP) causes signal sequence-dependent and site-specific arrest of chain elongation that is released by microsomal membranes. J. Cell Biol. 91: 557-561.

Walter P., Ibrahimi I. and Blobel G. 1981. Translocation of proteins across the endoplasmic reticulum I. Signal recognition protein (SRP) binds to *in vitro* assembled polysomes synthesizing secretory protein. J. Cell Biol. 91: 545-550.

Wang L. and Dobberstein B. 1999. Oligomeric complexes involved in translocation of proteins across the membrane of the endoplasmic reticulum. FEBS Lett. 457: 316-322.

Ware F., Vassilakos A., Peterson P., Jackson M., Lehrman M. and Williams D. 1995. The molecular chaperone calnexin binds $Glc_1Man_6GlcNAc_2$ oligosaccharide as an initial step recognizing unfolded glycoproteins. J. Biol. Chem. 270: 4697-4704.

Werner E. D., Brodsky J. L. and McCracken A. A. 1996. Proteasome-dependent endoplasmic reticulum associated protein degradation: An unconventional route to a familiar fate. Proc. Natl. Acad. Sci. USA 93: 13797-13801.

Whitley P., Nilsson I. and von Heijne G. 1996. A nascent secretory protein may traverse the ribosome/endoplasmic reticulum translocase complex as an extended chain. J. Biol. Chem. 271: 6241-6244.

Wiedmann B., Sakai H., Davis T. and Wiedmann M. 1994. A protein complex required for signal-sequence-specific sorting and translocation. Nature 370: 434-440.

Wienhues U., Becker K., Schleyer M., Guiard B., Tropschug M., Horwich A. L., Pfanner N. and Neupert W. 1991. Protein folding causes an arrest of preprotein translocation into mitochondria *in vivo*. J. Cell Biol. 6: 1601-1609.

Wiertz E. J. H. J., Jones T. R., Sun L., Bogyo M., Geuze H. J. and Ploegh H. J. 1996a. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. Cell 84: 769-779.

Wiertz E. J. H. J., Tortorella D., Bogyo M., Yu J., Mothes W., Jones T. R., Rapoport T. and Ploegh H. J. 1996b. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature 384: 432-438.

Wachter C., Schatz G. and Glick B. 1994. Protein import into mitochondria: the requirement for external ATP is precursor-specific whereas intramitochondrial ATP is universally needed for translocation into the matrix. Mol. Bio. Cell 5: 465-474.

Wilkinson B., Critchley A. and Stirling C. 1996. Determination of the transmembrane topology of yeast Sec61p, an essential component of the endoplasmic reticulum translocation complex. J. Biol. Chem. 271: 25590-25597.

Wilkinson B., Esnault Y., Craven R., Skiba F., Fieschi J., Képès F. and Stirling C. 1997. Molecular architecture of the ER translocase proped by chemical crosslinking of Sss1p to complementary fragments of Sec61p. EMBO J. 16: 4549-4559.

Vogel J., Misra L. and Rose M. 1990. Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. J. Cell Biol. 110: 1885-1895.

Voisine C., Craig E. A., Zufall N., von Ahsen O., Pfanner N. and Voos W. 1999. The protein import motor of mitochondria: Unfolding and trapping of preproteins are distinct and separable functions of matrix Hsp70. Cell 97: 565-574.

Wolin S. and Walter P. 1989. Signal recognition particle mediates a transient elongation arrest of preprolactin in reticulocyte lysate. J. Cell Biol. 109: 2617-2622.

von Heijne G. 1985. Signal sequences. J. Mol. Biol. 184: 99-105.

von Heijne G. 1990. The signal peptide. J. Membrane Biol. 115: 195-201.

Voos W., Gambill B. D., Guiard B., Pfanner N. and Craig E. A. 1993. Presequence and mature part of preproteins strongly influence the dependence of mitochondrial protein import on heat shock protein 70 in the matrix. J. Cell Biol. 123: 119-126.

YaDeau J., Klein C. and Blobel G. 1991. Yeast signal peptidase contains a glycoprotein and the *SEC11* gene product. Proc. Natl. Acad. Sci. USA 88: 517-521.

Zapun A., Darby N., Tessier D., Michalak M., Bergeron J. and Thomas D. 1998. Enhanced catalysis of ribonuclease B folding by the interaction of calnexin or calreticulin with ERp57. J. Biol. Chem. 273: 6009-6012.

Zapun A., Petrescu S., Rudd P., Dwek R., Thomas D. and Bergeron J. 1997. Conformation-independent binding of monoglucosylated ribonuclease B to calnexin. Cell 88: 29-38.

Zheng T. and Nicchitta C. 1999. Structural determinants for signal sequence function in the mammalian endoplasmic reticulum. J. Biol. Chem. 274: 36623-36630.

Zhu X., Zhao X., Burkholder W., Gragerov A., Ogata C., Gottesman M. and Hendrickson W. 1996. Structural analysis of substrate binding by the molecular chaperone BiP. Science 272: 1606-1614.