FLUORESCENT-DETECTED RETROTRANSLOCATION OF AN
ENDOPLASMIC RETICULUM - ASSOCIATED DEGRADATION (ERAD)
SUBSTRATE IN A MAMMALIAN IN VITRO SYSTEM

A Dissertation

by

JUDIT WAHLMAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Biochemistry
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Approved by:

Chair of Committee, Arthur E. Johnson
Committee Members, C. Nick Pace, Michael Polymenis, Gregory D. Reinhart
Head of Department, Gregory D. Reinhart

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ABSTRACT

Fluorescent-Detected Retrotranslocation of an Endoplasmic Reticulum-Associated Degradation (ERAD) Substrate in a Mammalian In Vitro System. (December 2007)

Judit Wahlman, B.S., University of Western Ontario

Chair of Advisory Committee: Dr. Arthur E. Johnson

Secretory proteins that are unable to assemble into native proteins in the endoplasmic reticulum (ER) are transported back into the cytosol for degradation. Many cytosolic and ER resident proteins have been identified so far as being involved in this retrotranslocation process, but it is difficult to determine whether these proteins have a direct or indirect effect. Interpretations are further complicated if the loss of a specific protein is obscured by the presence of another protein that is partially or wholly redundant. To overcome these limitations, a mammalian in vitro system was developed that allowed to monitor retrotranslocation synchronously and in real time in the absence of concurrent translocation.

To examine the roles of different components in ER-associated degradation (ERAD), well-defined and homogeneous mammalian ER microsomes were prepared biochemically by encapsulating a fluorescent-labeled ERAD substrate with specific lumenal components. After mixing ATP, specific cytosolic proteins, and specific fluorescence quenching agents with microsomes, substrate retrotranslocation was initiated. The rate of substrate efflux from microsomes was monitored spectroscopically
and continuously in real time by the reduction in fluorescence intensity as the fluorescent substrates passed through the ER membrane and were exposed to the quenching agents. Retrotranslocation kinetics were not significantly altered by replacing all lumenal proteins with only protein disulfide isomerase, or all cytosolic proteins with only the 19S proteasome cap. Retrotranslocation was blocked by affinity-purified antibodies against Derlin1, but not by affinity-purified antibodies against Sec61α or by membrane-bound ribosomes. Since the substrate also photocrosslinked Derlin1, but not Sec61α or TRAM, retrotranslocation of this ERAD substrate apparently involves Derlin1, but not the translocon. By labeling either the C- or N-terminus, it was revealed that the N-terminus of one ERAD substrate leaves the ER lumen first. This finding suggests that the protein is retrotranslocated as a linear polymer in a preferred direction.

When RRM s were reconstituted with a fluorescent-labeled ERAD substrate and various ions. Ca^{2+} ions in the ER lumen increased the rate and extent of retrotranslocation, while Ca^{2+} ions in the cytosol decreased retrotranslocation. This approach therefore provides the first direct evidence of the involvement and importance of specific ionic requirements for ERAD.
DEDICATION

To my two nieces, Jázmin and Véda,

“May you see your future as one filled with promise and possibility. May you find enough inner strength to determine your own worth by yourself, and not be dependent on another's judgment of your accomplishments. May you always feel loved.”

To my brother, Vince,

“May he find strength to face tomorrow in the love that surrounds him today.”
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Arthur E. Johnson, for having faith in my scientific ability by assigning me to a very challenging project and giving me the freedom to explore many areas of my research project. I thank him for being a great boss and a friend. I would like to thank my committee members, Dr. C. Nick Pace, Dr. Michael Polymenis, Dr. Gregory D. Reinhart for their guidance and support throughout the course of this research. I would like to thank everyone in the Johnson lab who taught me and provided for me throughout my graduate work. Particularly, I would like to thank Yiwei Miao and Yuanlong Shao who provided me with materials I needed to perform my experiments. Thanks also go to the Department of Biochemistry and Biophysics faculty and staff for making my time at Texas A&M University a great experience.

I would also like to thank Dr. John and Donna Trevithick for all their help and support during my undergraduate years in Canada. I thank my aunt Edit, and my friend Simon, for making all of this possible for me.

Thanks to my mother and my late father for giving me life and support. I would like to thank to my late grandmother for her encouragement and love during all her life. I thank Suraj, who has been my partner, best friend and colleague during the last six years.
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CHAPTER I

INTRODUCTION

Protein sorting at the endoplasmic reticulum membrane

In eukaryotic cell, genes located on a double-stranded DNA in the nucleus encode the vast majority of cellular proteins. According to Crick’s central dogma, RNA is made from the DNA template by transcription and protein from the messenger RNA by translation. Proteins are composed of amino acids and each of them is encoded by three base pairs. There are twenty amino acids and sixty-four possible code words, out of which three are stop codons (Palade, 1975). Proteins are synthesized by ribosomes, which are two-thirds RNA and one-third protein. There is a small and a large subunit in each ribosome, where the small subunit binds the mRNA and the large subunit contains two binding sites for tRNA. The transfer tRNA carries the appropriate amino acid that will be attached to the growing polypeptide when the anticodon of the tRNA recognizes the codon on the mRNA (Moore and Steitz, 2003).

Most protein synthesis occurs in the cytosol of the cell and the synthesized protein either remains cytosolic or directed to a specific compartment. For example, both secretory and membrane proteins are targeted to the endoplasmic reticulum (ER) (Berridge, 2002). These proteins are transported across or integrated into the ER membrane at sites called translocons.

This dissertation follows the style of Cell.
Figure 1. Schematic representation of a eukaryotic cell. Living organisms are composed of cells, which typically range from 5-30 µm in animals and 10-100 µm in plants. The eukaryotic cell encloses several organelles with distinct functions that are separated from one another by a tightly sealed lipid bilayer. These compartments include the cytosol, smooth and rough endoplasmic reticulum (ER), Golgi complex, nucleus, mitochondrion, free ribosomes, peroxisome, and lysosome. The figure was downloaded from http://nobelprize.org/nobel_prizes/medicine/laureates/1999/illpres/index.html.
The endoplasmic reticulum

The ER is an important eukaryotic cell compartment with specialized folding apparatus (Fig. 1). While mitochondria and chloroplasts may have evolved by endosymbiosis, the periplasm of gram-negative bacteria is perhaps the precursor of the ER. The periplasm of the gram-negative bacteria performs the folding and processing of the outer membrane proteins. Hence, it contributes to the maintenance of the outer membrane that separates the bacteria from its surroundings (Keenan et al., 2001). The ER similarly can be termed as a protein folding and processing factory of those proteins that reside in the plasma membrane, or in membranous organellar compartments, or are secreted into the extracellular medium.

In yeast, the ER processes almost one-third of the total proteins synthesized (Ghaemmaghami et al., 2003). These proteins eventually leave the ER (unless they are resident ER proteins) through the secretory pathway (ER-Golgi-plasma membrane) (Figs. 1 and 11) (Helenius et al., 1992). The ER is also the organelle where most of the membrane lipids are made for the cell (de Kroon et al., 2003). These newly-synthesized lipids and properly-folded proteins normally depart from the ER by vesicular transport through the Golgi to their final destination (Watson and Stephens, 2005). Mitochondria may receive its membrane lipids through direct contact sites with the ER (Schneiter, 2007). Other organelles, such as peroxisomes, might obtain some of their membrane proteins through the ER (Fig. 1) (Geuze et al., 2003).
Targeting to the ER membrane

Proteins have to traverse lipid bilayers to travel from one cellular compartment to another. This process is usually accomplished via a membrane protein complex, the translocation or translocon pore, which is an aqueous channel (Johnson and van Waes, 1999). Newly-synthesized mammalian proteins enter the ER lumen or integrate into the ER membrane via the translocon co-translationally, at the same time that the protein is being synthesized by a membrane-bound ribosome. However, yeast imports the majority of its proteins post-translationally into the ER.

Proteins are directed to the translocon by a 17-35-residue signal sequence at the N-terminus. The largely hydrophobic signal sequence in a ribosome-nascent chain complex (RNC) binds to the signal recognition particle (SRP) (Flanagan et al., 2003), which in turn binds to the SRP receptor in the ER membrane. The SRP-SRP receptor interactions therefore target the signal sequence-containing RNC to a translocon in the ER membrane. Both the SRP and the SRP receptor are GTPases, and upon GTP hydrolysis the SRP releases the signal-sequence. Following nascent chain transferal to the translocon, the nascent protein translocates into the ER lumen or integrates into the membrane while it is still being synthesized by a ribosome (Fig. 2) (Egea et al., 2005; Keenan et al., 2001; Walter et al., 2000).
Figure 2. Current view of co-translational protein synthesis at the ER membrane. When the signal sequence emerges from the ribosome, it binds to the signal recognition particle (SRP). The SRP-ribosome complex is then targeted to the SRP receptor and the ribosome docks on the translocon. The nascent polypeptide is transferred to the translocon and translocated into the ER lumen and the SRP dissociates from its receptor following GTP hydrolysis. The signal peptide is cleaved off the growing nascent chain in the ER lumen by the signal peptidase (SP). The figure was downloaded from http://nobelprize.org/nobel_prizes/medicine/laureates/1999/illpres/index.html.

The translocon

Many scientific studies have examined the molecular mechanism of translocation through the ER membrane. These studies have established that the translocation process involves a core heterotrimeric membrane protein complex that is well conserved from bacteria to human (Johnson and van Waes, 1999). The translocon forms an aqueous channel that allows the newly synthesized protein to pass through the ER membrane (Crowley et al., 1994).
The largest component of the heterotrimeric Sec61/SecY translocon is the α-subunit, named Sec61α in higher eukaryotes, Sec61p in yeast, and SecY in bacteria. This protein has ten transmembrane segments, and both of its termini are in the cytosol. The β-subunit with one transmembrane segment is called Sec61β in mammals, and Sbh1p in yeast, Secβ in archaea, while SecG in eubacteria with two transmembrane segments. The γ-subunit spans the membrane once and is called Sec61γ in mammals, Sss1p in yeast, and SecE in eubacteria and archaea (Osborne et al., 2005).

**Calcium homeostasis in the ER**

A dynamic Ca\(^{2+}\) storage and cell signaling are amongst the many roles of the ER (Berridge, 2002). The ER can receive and release multiple signals, including intracellular messengers such as Ca\(^{2+}\), inositol 1,4,5- triphosphate, reactive oxygen species, sphingosine-1-phosphate, and sterols (Gorlach et al., 2006). For example, the ER participates in removing excess Ca\(^{2+}\) ions from the cytoplasm after cellular activation (Verkhratsky and Petersen, 2002). The concentration of Ca\(^{2+}\) in the ER lumen is a thousand times greater then in the cytoplasm (Ca\(^{2+}\)_ER : 100-800 µM, Ca\(^{2+}\)_Cytosol : 0.1-1 µM) (Fig. 3) (Demaurex and Frieden, 2003; Mogami et al., 1999) (Fig. 3).

The major functions of the ER influence each other, since an increase in the amount of misfolded proteins within the ER can change Ca\(^{2+}\) homeostasis, while variations in the lumenal Ca\(^{2+}\) content can disturb proper protein synthesis (Burdakov et al., 2005). The role of Ca\(^{2+}\)- binding proteins in the ER is important, since some of them (calreticulin, calnexin, BiP etc.) require Ca\(^{2+}\) for their activity (Llewelyn Roderick et al., 1998). If the [Ca\(^{2+}\)] falls below ~50 µM in the ER lumen, the chaperone activity of these proteins may
be completely inhibited (Corbett and Michalak, 2000). Disturbance in Ca\(^{2+}\) homeostasis can trigger ER stress that ultimately can result in cell death (Groenendyk and Michalak, 2005). Additionally, the majority of ER chaperones and folding enzymes have a low affinity and high capacity for Ca\(^{2+}\) ions thereby serving as a passive storage (Brostrom and Brostrom, 2003). When Ca\(^{2+}\) ions were depleted from the ER, glycoprotein processing was inhibited. These glycoproteins remained in the high mannose configuration (Cooper et al., 1997). The unprocessed glycoproteins are then recognized by the ER quality control apparatus and retained within the ER. If the proper Ca\(^{2+}\) content is restored in these cells, these retained proteins can traffic towards the Golgi (Helenius and Aebi, 2004; Parodi, 2000). Relatively little is known about the requirements for Ca\(^{2+}\) ions in the folding of nonglycosylated proteins.

There are multiple ways that Ca\(^{2+}\) can exit and enter the ER. Ca\(^{2+}\) is released via either inositol 1,4,5-triphosphate receptors (InsP3Rs) or ryanodine receptors (RYRs) (Varadi and Rutter, 2004) (Fig. 3). The translocon itself may participate in passive Ca\(^{2+}\) release from the lumen over time (Flourakis et al., 2006; Van Coppenolle et al., 2004), although in vitro fluorescence quenching experiments proved that it is not the case (Hamman et al., 1998). The translocon should be able to move chemically and sterically different proteins across the ER membrane while preserving the permeability barrier contrary to ion-channels that allow specific ions transport (Crowley et al., 1994).
Figure 3. Various mechanisms of Ca\(^{2+}\) ion regulation inside and outside of the ER.

Ribosomes have to form an ion tight seal with the translocon on the cytosolic side to prevent small ions entering in and out of the ER during co-translational translocation (Johnson, 2003).

One of the most important regulators of the ER-located Ca\(^{2+}\) release channels is Ca\(^{2+}\) itself (Kang and Park, 2005). When the Ca\(^{2+}\) concentration increases in the ER lumen over the buffering capacity of the ER, these channels (RyRs and InsP3Rs) become more likely to be open (Xu et al., 2000). The Ca\(^{2+}\) from the ER lumen can also be depleted by
chelating agent, response to hormones, or various SERCA pump inhibitors (Ferzandi and MacGregor, 1997).

Ca\(^{2+}\) ions enter the ER via Sarco (Endo) plasmic Reticulum Ca\(^{2+}\) ATPases (SERCA) pump (Vangheluwe et al., 2005) (Fig. 3). When the Ca\(^{2+}\) level in the ER declines, it can be replenished through SERCA2b; hence a constant Ca\(^{2+}\) level is maintained in the ER (Berridge et al., 2003). Decrease in [Ca\(^{2+}\)]\(_{ER}\) can increase the velocity of Ca\(^{2+}\) uptake by SERCA pumps (Yano et al., 2004). ER chaperones, calnexin, calreticulin and Erp57, have been shown to interact physically with SERCA 2b in a Ca\(^{2+}\) dependent manner (John et al., 1998; Li and Camacho, 2004). Erp57 causes disulfide bond formation in the L4 region of the SERCA 2b pump that slows its Ca\(^{2+}\) pumping (Li and Camacho, 2004). TRAM2, TRAM homologue with an unclear function that is associated with the ER translocon directly interacts with SERCA 2b by its C-terminus (Stefanovic et al., 2004). It was suggested that this contact has an important regulatory role in maintaining a high Ca\(^{2+}\) concentration in the ER during protein synthesis. This interaction also links the translocon to the Ca\(^{2+}\) concentration in the ER (Stefanovic et al., 2004).

**General overview of protein folding**

Proteins must fold properly into their three-dimensional structure in order to fulfill their biological functions. For short proteins, the protein primary sequence and its environment determine its final structure and folding pathway, for longer proteins, individual domains may fold independently before assembling into the final structure. Proteins often have different conformational states that result in different functional states. When a protein reaches a stable conformational state via different folding
intermediates, this state is typically presumed to have the lowest potential energy, and is called the native structure (Bryngelson et al., 1995). This lowest potential energy model implies that protein folding may reach the final conformation without external energy input or additional factors, but this pathway may only apply to small proteins (Anfinsen and Scheraga, 1975; Jaenicke, 1991a; Jaenicke, 1991b). Protein folding is sometimes slow and inefficient, because partially-folded off pathway intermediates can also fold into a low energy states that may have long lifetimes (Daggett and Fersht, 2003a; Daggett and Fersht, 2003b). The possibility of such states increases as the length of the proteins increases, thus proteins can fold in vitro without help, but the in vivo folding of proteins, especially long proteins may require chaperones to reduce incorrect folding or irreversible aggregation (Gething and Sambrook, 1992).

Because of the deleterious effects of protein misfolding in the cell, chaperones and folding factors are amongst the most abundant proteins in the cell, and they frequently conserved from bacteria to eukaryotes (Ellenberg et al., 1997). In contrast to prokaryotes, eukaryotic cells contain several organelles surrounded by lipid bilayers (Fig. 1). To enter such an organelle, proteins usually cross the lipid membrane in an unfolded linear state (Johnson and van Waes, 1999). These proteins then fold inside the organelle assisted by the folding machinery specific for the compartment (mitochondria, ER, or chloroplast in plants).

**Protein folding in the ER**

The ER is a compartment that has been evolved to facilitate complex protein folding, modification and oligomerization. The ER lumen contains soluble proteins at
concentrations higher than 100 mg/ml concentration of proteins. This crowded and dense proteinaceous environment must have a well-organized and methodical system in order to carry out accurate protein folding and avoid protein aggregation (Marquardt et al., 1993). This is important, since hydrophobic elements of newly-synthesized proteins are exposed (Stevens and Argon, 1999; Tu and Weissman, 2004). As a result, the ER lumen contains many proteins that function as molecular chaperones to promote folding and prevent aggregation (Fig 4) (van Anken and Braakman, 2005).

The ER also differs from other organelles by an oxidizing environment and a unique glycosylation machinery (Kowarik et al., 2002; Molinari and Helenius, 2000). Many posttranslational modifications, occur in the ER lumen, such as glycosylation, disulfide bond formation, proline isomerization, and GPI anchor addition (Kleizen and Braakman, 2004).

ER resident enzymes and chaperones that assist the folding include BiP, an Hsp70 chaperone (Kar2p in yeast) with its co-chaperones (Hsp40), PDI and PDI-related proteins that belong to the protein disulfide isomerase family, and members of peptidyl-prolyl isomerase family (Fig. 4). Many chaperones that play a role in N-glycosylation have been identified up to this date. These include calnexin (Cne1p in yeast), calreticulin, EDEM (Htm1p in yeast), and OS-9 (Yos9p in yeast). Several enzymes are involved in removing/adding glucose and mannose residues back to the carbohydrate moiety (Ruddock and Molinari, 2006) (Fig. 4).
Figure 4. ER resident chaperones and folding enzymes. Proteins in the ER are sorted according to their protein families. The homologues between yeast and mammalian proteins are shown in the same row, and other common names are listed there as well. This table was adapted from (van Anken and Braakman, 2005).

<table>
<thead>
<tr>
<th>Family</th>
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<tr>
<td>Mannosidase I</td>
<td>ER α 1,2-mannosidase</td>
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Lectin, glycan trimming enzymes etc.
CNX/CRT | Calnexin | Cne1p
UGGT | UGGT | |
EDEM | EDEM1 | Hm1p
EDEM3 | |
Glucosidase I | Glucosidase I | |
Glucosidase II | Glucosidase II α | Glc1p
abbr | Glucosidase II β | Glc2p
abunit | |
Mannosidase I | ER α 1,2-mannosidase | |
Mns1p | |
Protein disulfide isomerase (PDI)

PDI was first identified in 1963 by Anfinsen’s group as a protein that catalyzes oxidative folding in vivo. PDI has two important functions in the ER (Goldberger et al., 1963). The first is to catalyze the formation or reshuffling of disulfide bonds, and the other is to bind unfolded proteins to help folding, and thereby act as a molecular chaperone (Bardwell et al., 1991). PDI is one of the most abundant proteins in the cell, comprising almost 0.8% of total cellular proteins both in yeast and mammalian cells (Ferrari and Soling, 1999). It is a member of the thioredoxin (TRX) family that has a common motif of WCGHCK in its two active sites (Martin, 1995) (Fig. 5A). The two amino acids positioned between the two cysteines in the characteristic CXXC catalytic motif of the thioredoxin family members usually determine the redox potential (Ellgaard and Ruddock, 2005). The thioredoxin protein family also includes other ER residents, namely Erdj5, PDlp, Erp57, Erp44, Erp72, PDIR, and P5 in mammals and Eug1, Mpd1 and Mpd2 in yeast (Fig. 4).

The recently reported crystal structure of the yeast PDI sheds light on oxidative folding in more details (Tian et al., 2006). The four thioredoxin domains in the yeast and mammalian PDI are arranged in a bent ‘U’ shape (a, b, b’, a and c, where the a, a’ and b, b’ domains contain the thioredoxin sites) (Fig. 5B). The catalytically active regions are located in a and a’ domains with a pKa around 4.5 (the free cysteine is has a pKa near 8.5) that makes them a highly reactive thiol-group (Freedman et al., 1994) (Figs. 5A and B). The redox potential of PDI usually determines the type of reactions it catalyzes (Wilkinson and Gilbert, 2004).
Figure 5. The domain organization of PDI and the oxidative protein folding apparatus in the eukaryotic ER. (a) On the basis of various models of human PDI and the crystal structure of yeast PDI (S. cerevisiae, PDB ID code 2B5E), the architecture of PDI consists of five domains and one linker organized in the order abb′xa′c. Residues are numbered according to mature human PDI (H. sapiens, SwissProt code P07237). Domain boundaries are based on the yeast crystal structure, and border residues and the two active-site domains (shown in detail in the boxes) are aligned for human (top) and yeast (bottom) PDI. Domains a (orange) and a′ (yellow) are homologous to TRX and contain the catalytic CxxC motif (green). Domains b (dark blue) and b′ (light blue) also adopt a TRX fold, but do not share high sequence similarity with each other or with domains a or a′. The flexible linker region x (black) is located between domains b′ and a′. The link between domains a and b is only one residue. The C-terminal extension (red) contains a (K/H)DEL retention signal for the ER. (b) Ribbon diagram based on the crystal structure of yeast PDI showing the active-site cysteines in green space-filling representation (prepared with Molscript. Colors of the domains are the same as in (a). This figure is reprinted with permission from Elsevier (2007) (Gruber et al., 2006). (c) PDI with oxidized disulfide bonds (ii. bottom, only one of the PDI active sites is shown) participates in the oxidative folding of a reduced substrate (i. bottom). Then the reduced PDI (ii. top) can act as an isomerase to reshuffle the disulfide bonds of the substrate in order to form the correct ones (i. top). PDI becomes oxidized again via the Ero1 cascade (iii. and iv.). Electrons are donated from the reduced PDI (ii. middle, iii. top) to Ero1 flexible loop, which is located outside of Ero1. Subsequently these electrons flow through the rigid loop of Ero1 (located in the interior of Ero1) and the cofactor flavin adenine dinucleotide (FAD) to eventually molecular oxygen. This figure was adapted from (van Anken and Braakman, 2005).
The reactive thiol groups are solvent-exposed, and the reaction begins by a nucleophilic attack on the cysteine residue of the unfolded protein to form mixed disulfide bonds between PDI and the substrate (Fig. 5C). Thioredoxins must have their two active sites in close proximity of each other to exert isomerase activity. This way the conformational constrains are minimal when the two active sites simultaneously break the incorrect disulfide bonds to create a correct one.

It has been shown that PDI binds to unfolded proteins preferentially, due to its continuous hydrophobic surface on b and b’ domains (Freedman et al., 2002; Klappa et al., 1998; Noiva et al., 1993) (Fig. 5B). This hydrophobic patch can accommodate a wide variety of unfolded proteins, regardless of the position of their disulfide bonds. For
the PDI to be catalytically active, it has to bind many different folding intermediates with low to moderate affinity and be able to release them after the catalytic cycle has been completed.

When proteins with newly-formed disulfide bonds leave the ER, an equal number of electrons remain in the ER. These electrons pass first to PDI and in yeast, eventually to the ER oxidoreductin 1 protein (Ero1p) (Fig. 5C) (Tu and Weissman, 2004). Ero1p
shuffles electrons from its cofactor FAD, which becomes reduced to FADH$_2$ (Tu et al., 2000). These electrons eventually pass to molecular oxygen and create a reactive oxygen species (ROS). There are two different Ero1 [Ero1$\alpha$ (Cabibbo et al., 2000) and Ero1$\beta$ (Pagani et al., 2000)] in mammalian cells. These two forms may have similar roles and contact PDI directly (Benham, 2005). There is another yeast protein (Erv2p) that seems to have a function analogous to Ero1p (Gerber et al., 2001). The Ero1p crystal structure reveals a CXXC thioredoxin domain with a bound FAD that acts a rigid structural element. Ero1p has another disulfide bond motif (CXXXXC) that is on a flexible loop, and might transfer the electrons from PDI to the rigid CXXC motif (Gross et al., 2004). The formation of native disulfide bonds in the substrate is energetically more favorable, so mixed disulfide bonds are broken faster and reoxidized by PDI. Experimental evidence suggests that individual PDI domains (a or a’) are able to oxidize or reduce disulfide bonds, but both domains are necessary for isomerization (Darby and Creighton, 1995). The redox activity of the catalytic domains varies within organisms (Wilkinson et al., 2005).

Bacteria have also PDI analogues in its periplasmic space with very little sequence homology to the mammalian PDI. These proteins are called DsbA (Martin et al., 1993), DsbE (an oxidase) (Edeling et al., 2002) and DsbB and C (reductase and isomerase) (Fig. 6) (McCarthy et al., 2000).

DsbA, a reduced soluble periplasmic protein oxidizes the newly synthesized and translocated proteins (Guddat et al., 1998) (Fig. 6). The oxidized DsbA is reduced back by DsbB, an inner membrane protein. DsbB utilizes the electron transport chain to
donate an electron to molecular oxygen via a quinone and cytochrome oxidases (Gruber et al., 2006). Under anaerobic conditions the electron is donated to either nitrate reductase or fumarate reductase. DsbC and DsbD together facilitate isomerization. DsbD, a membrane protein receives an electron from NADPH through a thioredoxin located in the cytoplasm (Rozhkova et al., 2004). The DsbC in turn reintroduces correct disulfide bonds into proteins with incorrect disulfide bonds. The bacterial DsbC bears structural similarity to the yeast PDI, forming a homodimer with a ‘V’ shape, where the active sites are located at the ends of the ‘V’. Hydrophobic residues are located inside the ‘V’, which can facilitate the binding of unfolded or misfolded proteins (McCarthy et al., 2000).

There are differences between eukaryotic and bacterial thioredoxin, including the distances between the redox domains. In eukaryotic cells, the two catalytic sites are separated by ~26 Å and gated two tryptophan residues; while in bacterial DsbC and DsbG these sites are 40 Å and 60 Å apart, respectively, and more accessible to substrates (Figs. 6B and 7) (Gruber et al., 2006).

The Hsp70 family

This heat shock protein (Hsp) family participates in many cellular processes that include folding and assembly of newly synthesized proteins, facilitating post-translational translocation on both sides of the ER membrane, maintaining permeability barrier of the mammalian ER (Hamman et al., 1998), unfolding and refolding of misfolded proteins, regulating the activity of its co-chaperones [J-domain proteins, nucleotide exchange factors (NEF)] (Bukau and Horwich, 1998). The Hsp70 family has
Figure 7. Characteristics of the Hsp70 catalytic cycle. The nucleotide-binding domain (NBD) is depicted in light blue; the substrate-binding domain (SBD) is in yellow. J-domain proteins stimulate ATP-hydrolysis and thereby substrate binding by SBD. The nucleotide exchange factor (NEF) stimulates the exchange of ADP to ATP and the release of the substrate from SBD. This figure was adapted from (Bukau et al., 2006).

also evolved into various distinct chaperones; such as the Hsp100 and Hsp170 (Fig. 4) (Bukau et al., 2000).

Hsp70 chaperones contain a substrate-binding domain (SBD) and a nucleotide-binding domain (NBD) (Fig. 7). SBD has a high affinity for the hydrophobic stretches that are usually exposed in an unfolded or misfolded protein (Bukau et al., 2006). SBD and NBD are allosterically coupled. ATP binds to the N-terminal NBD and thereby
causes the substrate-binding pocket with its helical lid to open up and interact with the substrate. J-domain proteins can promote ATP hydrolysis, and thus indirectly trigger the closing of SBD and the trapping of the substrate there (Jiang et al., 2005).

The NBD consists of two globular subdomains that are connected via the nucleotide-binding groove, while the SBD contains a β-sandwich with one α-helix (Helix A) on its side and another (Helix B) on the top to serve as a lid for the substrate binding groove. A ten-residues-long conserved linker (VQDLLLLDV) connects NBD and SBD the signals between the two domains (Jiang et al., 2005; Wei et al., 1995). The crystal structure also revealed that Helix A is embedded in the NBD cleft directed thereby ionic, and relays interactions at the C-terminal end of the Helix A and hydrophobic interactions at the N-terminal end (Jiang et al., 2005). An additional contact between these two subunits is made by the β-sandwich and the NBD cleft. These interactions are probably disrupted upon ATP binding to NBD, and hence aid the opening of the SBD. Structural changes within the NBD also accompany ATP hydrolysis and probably transmitted to the SBD through a hydrogen-bonding network, as in the case of bacterial DnaK (Vogel, Bukau et al. 2006).

By themselves Hsp70s act very slowly, thus they usually employ co-chaperones. Numerous J-domain proteins have been identified in yeast (Walsh et al., 2004). The role of these J-domain chaperones is to coordinate ATP hydrolysis and substrate binding by Hsp70. The affinity of Hsp70 is very high for ADP, so the dissociation is slow without a nucleotide exchange factor. Several NEFs have been discovered to date, and their functions are important to promote the release of ADP and rebind ATP (bacterial GrpE,
eukaryotic BAG proteins, yeast cytosolic Fes1p and lumenal Sls1p, human Hsp70 binding protein 1). The considerable number of different Hsp70s and their interactions with a variety of NEF and J-domain proteins suggest a wide range of substrate specificity and a capability to participate in different cellular events.

**N-glycosylation in the ER**

Our understanding of the N-glycosylation process in the ER lumen has increased considerably during the past decade (Helenius and Aebi, 2001; Ruddock and Molinari, 2006). N-linked oligosaccharide moieties have various cellular functions including increase solubility, determine orientation of the protein respect to the membrane, and regulate protein turnover, control immune responses and the isoelectric point of the proteins (Helenius and Aebi, 2004).

An ER translocon-associated protein, the oligosaccharyl transferase or OST, transfers a carbohydrate moiety via an N-glycosidic bond to the side chain nitrogen of an asparagine residue on nascent chain containing a glycosylation sequence (Asn-X-Ser/Thr) (Fig. 8) (Hebert et al., 2005). The side chains of threonine or serine thought to form a loop with the asparagine amine and therefore make the amine more nucleophilic. The yeast OST complex contains nine subunits (Ost1p, Ost2p, Wbp1p, Stt3p, Swp1p, Ost3p, Ost4p, Ost5p and Ost6p), homologues, which are also present in mammalian cells, and the first five subunits are essential for proper function (Yan and Lennarz, 2005). The STT3 subunit contains the OST active site and photocrosslinking experiments showed that glycosylation occurs when the Asn-X-Thr/Ser is located about
Figure 8. The structure of the core N-glycan. The core glycans have fourteen saccharides; three glucoses (red triangles), nine mannoses (green circles) and two N-acetylglucosamines (blue squares). The three mannose branches are named A, B and C. The trimming sites for mannosidase I and endomannosidase are shown by arrows. The saccharides from A-F and I are added on the cytosolic side of the ER membrane, while the rest is on the lumenal side after flipping. Residues D, F, L and I contribute to the interaction of calnexin and calreticulin with the monoglucosylated glycans. Saccharides E, G, H and K possibly interact with EDEM. This figure was adapted from (Helenius and Aebi, 2004).

75 residues away from the ribosomal P-site and hence in close proximity to the Sec61 complex (no more than 5 nm) (Nilsson et al., 2003).
N-glycans covalently attached to the protein are subject to modifications both in the ER and in the Golgi apparatus, as certain saccharides are trimmed and/or added. Why would a cell develop such an intricate process, and why is this process conserved among eukaryotes (Trombetta and Parodi, 2003)? The answer appears to be that each configuration during oligosaccharide processing has its own role. In the ER, these moieties are necessary for proper folding and to pass quality control (Helenius and Aebi, 2004). The N-glycans go through extensive modifications in the Golgi for intracellular transport, and eventually they exert their effect at the final destination (lysosomes, extracellular space, plasma membrane etc.) (Ruddock and Molinari, 2006) (Fig. 8).

The OST complex preferentially conjugates the complete glycan to the incoming nascent chain, even if it means underglycosylation (Burda and Aebi, 1999; Kornfeld and Kornfeld, 1985). N-glycans are located on the surface of the fully folded protein (Wormald et al., 2002) and protrude when hydrated about 30 Å into the solvent. A glycoprotein can contain several glycans, each with a molecular mass of about 3 kDa. In some cases, glycans can be removed from the protein surface without significantly affecting the protein structure (Helenius and Aebi, 2004).

The core glycan is synthesized in both the cytosol and ER through conjugation of individual saccharides to the lipid carrier dolichol-pyrophosphate by monosaccharyl transferases (Helenius et al., 2002). Seven carbohydrates are added on the cytosolic side followed by an ATP-independent flipping of the carbohydrate moiety to the lumenal side by bi-directional flippase (Hirschberg and Snider, 1987; Perez and Hirschberg, 1987). The final configuration is Glc$_3$Man$_9$GlcNAc$_2$ (Fig. 8).
The affinity of carbohydrate binding chaperones (lectins) for N-glycans is generally low (µM range), so only transient lectin-lectin interactions occur in the already crowded environment of the ER lumen (Kapoor et al., 2003; Patil et al., 2000). Two lectins participate in the folding of glycoproteins: calnexin (CNX) (Hammond et al., 1994; Hammond and Helenius, 1993), a type I membrane protein, and calreticulin (CRT) (Nauseef et al., 1995; Peterson et al., 1995), a soluble ER resident protein.

Glycoproteins typically go through a CNX/CRT folding cycle before they are secreted. In this cycle, after covalent addition of the core glycan, two glucose-cleaving enzymes [glucosidase I (GI) and glucosidase II (GII)] sequentially remove one glucose residue to create Glc¹Man₉GlcNAc₂ (Elbein, 1983; Trombetta et al., 1996). Inhibitors of ER glucosidases block the interactions between CNX and the N-glycans (Hammond and Helenius, 1994), confirming that only mono-glucosylated species interact with either CNX or CRT through their carbohydrate moiety. CNX and CRT serve as molecular chaperones in association with a cofactor, Erp57, a protein-disulfide isomerase that catalyzes the formation of disulfide bonds (Frickel et al., 2004; Frickel et al., 2002). GII has to remove the last glucose residue for the folding intermediate to be released. If the protein is properly folded, then now it is ready to be secreted towards the Golgi.

Glucosyltransferase (UGGT) identifies misfolded proteins and a glucose is added back to its original position to re-initiate a folding attempt by CNX/CRT (Sousa et al., 1992).
**Calnexin and calreticulin**

These lectins are both monomeric, bind calcium, and have ER retention signals (Bouvier and Stafford, 2000). They have two distinct domains; a β-sandwich, globular domain and a proline-rich region (P-domain) (Ellgaard et al., 2001a; Ellgaard et al., 2001b; Schrag et al., 2001) (Fig. 9A) Calnexin (CNX) also contains a membrane spanning region and a cytosolic segment that can undergo phosphorylation and interact with the ribosome (Chevet et al., 1999). The x-ray crystal structure of CNX revealed that a glycosylated substrate protein could reside in a cavity between the P-domain and the globular domain, while its glycan could bind simultaneously to the lectin-binding site in the globular domain (Fig. 9B). This pocket is flexible enough to adapt to the structure of a folding polypeptide, and hence preventing premature aggregation. Erp57 also weakly interacts with the end the P-domain (Ellgaard et al., 2001b). Both CNX and calreticulin (CRT) have the same specificity: an α-1,3 linked glucose that is conjugated to three mannoses (α-chain) (Ware et al., 1995). The formation of hydrogen bonds between asparagine and tyrosine and the glucose hydroxyl group is the reason for the preference for α-1,3 linked glucose (Kapoor et al., 2004). Three of the mannoses on branch A also contribute to the stability of N-glycan binding within the globular domain via additional controversial because most of the substrate proteins do not bind when the ER glucosidase activity is impeded (Danilczyk and Williams, 2001). Moreover, no hydrophobic patch has been identified in the protein-binding cavity that could serve as a nonspecific-binding site (Schrag et al., 2001). Some experimental evidence also
Figure 9. The structure of the calnexin ectodomain and the calreticulin oligosaccharide-binding site. (A) Domain structure of calnexin. The 3-D structure of calnexin comprises a lectin domain (blue) and an extended arm called the P-domain, which comprises four repeat modules, P1–P4 (cyan, green, yellow and red, respectively). Each repeats module is formed by an antiparallel interaction of two different proline-rich sequence motifs. The observed glucose-binding site in the lectin domain is indicated by a ball-and-stick model (green). Glc1Man9GlcNAc2 glycoprotein binding was modeled by superimposing the terminal glucose of the model glycan on the observed position of bound glucose. The resulting position of the polypeptide of the model glycoprotein (properly folded ribonuclease B in this example; magenta) suggests that interaction with the P-domain is likely. This figure is reprinted with permission from TiBS (Schrag et al., 2003). (B) A model of the oligosaccharide-binding site of calreticulin. The tetrasaccharide bound is Glcα1-3Manα1-2Manα1-2Man. Mutational analysis showed that several of the residues that interact with the sugar in the model are essential for binding. This figure is reprinted with permission from Annual. Rev. Biochem. (Helenius and Aebi, 2004)
suggested a possibility of ATPase activity also, although its function is not yet clear (Ou et al., 1995).

**Glucosidase I**

Glucosidase I (GI) is an 82 kDa single-spanning membrane protein (type II) with a short N-terminal cytosolic region and a substantial luminal domain that contains the glucosidase activity. GI facilitates glycoprotein movement from the OST complex by trimming the outermost $\alpha$-1,2 linked glucose, and after further trimming by glucosidase II (GII), passing the folding protein on to the CNX/CRT cycle (Helenius and Aebi, 2004).

**Glucosidase II**

GII is a soluble luminal protein composed of $\alpha$ and $\beta$ glycoproteins chains, 107 kDa and 54 kDa respectively that are tightly associated with each other (Trombetta et al., 1996). $\beta$-chain is a highly conserved protein with an N-terminal signal sequence and a C-terminal lectin domain with an ER retention signal. Its lectin domain is homologous to the mannose-6-P family receptors. The $\alpha$-chain does not contain a retention signal, so it has been speculated that its association with the $\beta$-chain serves to localize the protein in the ER. Both subunits are essential for the activity, stability and solubility of this enzyme (D'Alessio et al., 1999; Pelletier et al., 2000).

**UDP-glucose transferase (UDP-glucose:glycoprotein glucosyltransferase or UGGT)**

UGGT is a large, soluble luminal protein with an ER retention signal, and in addition to the ER lumen, it is also found at ER exit sites, and in the ER to Golgi intermediate compartment (ERGIC) (Parodi, 2000). The catalytic site resembles that of
the glycosyltransferase family members and it is located within the 300 amino-acid long C-terminus. The N-terminal might play a role in substrate recognition, and it is essential for C-terminal activity (Arnold and Kaufman, 2003; Guerin and Parodi, 2003). UGGT transfers a glucose residue from UDP-glucose to an N-glycan with a Man$_9$GlcNAc$_2$ configuration. It has the highest affinity for those N-glycans that contain all mannoses on the B and C chain (Fig. 8).

When mannoses are trimmed off, both GII and UGGT interact more slowly with a substrate (Sousa et al., 1992). This observation suggested that the loss of mannoses and a decrease in the folding speed might signal the terminal misfolding of the protein, and eventual targeting for degradation (Trombetta and Parodi, 2005). It was proposed that both glycan binding and interaction with the hydrophobic regions of the glycoprotein are necessary for UGGT activity (Sousa et al., 1992). UGGT has affinity for substrates such as molten globule-like glycoproteins, and glycopolypeptide chains that have at least 12 amino acids in hydrophobic patches (Ritter and Helenius, 2000; Ritter et al., 2005).

Some groups suggested that UGGT can interact with hydrophobic proteins without glycans, since UGGT preferentially reglucosylated glycoproteins that were present on misfolded regions of the protein, while leaving the ones on a fully folded domain intact (Helenius and Aebi, 2004).

**UDPase**

UDP-glucose is transferred from the cytosol into the ER lumen through an antiporter that couples its entry to the exit of UMP (Failer et al., 2002; Perez and Hirschberg, 1987). There are three different nucleoside diphosphatases (NDP) up to date; two are
located in the ER and one in the Golgi. One of the ER-localized NDPs is a membrane protein that requires Ca\(^{2+}\) ions for its proper functioning. Soluble NDPs need Ca\(^{2+}\), Mg\(^{2+}\), or Mn\(^{2+}\) for activity, and they hydrolyze UDP, GDP and IDP, but not any nucleoside triphosphate or ADP (Helenius and Aebi, 2004).

**Lectin homologues in the secretory pathway**

ERGIC-53, a broadly-expressed type I transmembrane protein, cycles between the ERGIC and the cis-Golgi compartments because of its ER retention signal. It belongs to the leguminous protein family with mannose lectin activity that contains a \(\beta\)-sandwich domain like CNX and CRT, but no P-domain (Velloso et al., 2002a; Velloso et al., 2002b). ERGIC-53 is responsible for transporting a certain set of glycoproteins that are destined for secretion or for delivery to the lysosome. It usually interacts with proteins that are successfully folded in the CNX/CRT cycle (Appenzeller et al., 1999).

Vesicular integral protein 36 (VIP 36) is also a membrane protein, homologous to ERGIC-53 (Fiedler et al., 1994). It interacts with high-mannose containing proteins that are targeted to the apical plasma membrane. There are several other lectin-binding proteins identified in the secretory pathway, and their role involves recognition, proper folding, quality control and transport of glycoproteins (Schrag et al., 2003).

**Mannosidases**

The removal of an \(\alpha\)-1,2 mannose from the B and the C chain (Fig. 8) is completed by Mannosidase I (ManI) and Mannosidase II (ManII), respectively, enzymes that are present in both mammalian and yeast (Fig. 4) (Dahms and Hancock, 2002). The mannose 6-phosphate binding domain forms a \(\beta\)-barrel structure that contains three
disulfide bonds. The binding groove is seated deep within this β-barrel, enclosing a Mn$^{2+}$ ion that interacts with a phosphate group in the carbohydrate. A conformational change in the receptor occurs upon ligand binding. This binding event is supported by a mildly acetic pH (Dahms and Hancock, 2002).

ManI belongs to a GH47 enzyme family, which consists of three subfamilies: ER α-mannosidase (ERManI) family, Golgi α-mannosidase family (GolgiManIA, IB and IC in mammals, but absent in yeast), and the putative ER lectins, ER-degradation-enhancing α-mannosidase-like protein (EDEM 1-3 in mammals and Htmp1/Mnl1 in yeast) (Henrissat and Davies, 1997; Hosokawa et al., 2001). Both ERManI and GolgiManI are Ca$^{2+}$ dependent enzymes with an (αα)$_7$-barrel structure with the active site in the center of the barrel. EDEM 1 has a 35% sequence identity to the catalytic domain of ManI, but has no mannose cleaving activity despite its conserved domain structure. EDEM lacks the disulfide bond in its mannosidase-like domain that may be necessary for activity (Molinari et al., 2003; Oda et al., 2003). However, EDEM might still act as a mannosidase, because many mannosidases do not require this disulfide bond for their function (Karaveg and Moremen, 2005; Karaveg et al., 2005; Mast et al., 2005). EDEM can be inhibited by both Kif and dMNJ, and upregulation of EDEM 1 and 3 results in an increased de-mannosylation in the cell (Hirao et al., 2006; Olivari et al., 2006). EDEM 1 is a type 2 membrane protein, with two soluble homologues (EDEM 2 and 3) (Oda et al., 2003). First, it was reported that its uncleaved signal sequence anchors EDEM 1 into the membrane, but it was later reported that the signal sequence is cleaved off and the
mature EDEM 1 enters the lumen in a soluble form challenged this finding (Oda et al., 2003; Olivari et al., 2005).

**ER quality control**

Soluble secretory proteins enter the ER lumen and membrane proteins integrate into the ER membrane in an unfolded state (Johnson and van Waes, 1999). Some of these proteins then diffuse into COPII vesicles that are directed to the Golgi apparatus where the proteins are sorted and trafficked to their final destination (intracellular organs, plasma membrane, vacuoles etc.) (Fig. 10) (Barlowe, 2002). Intracellular protein export occurs primarily through vesicular transport by vesicle budding off the donor membrane and ultimately fusing with the acceptor membrane (Barlowe, 2003). The Rab family proteins, which are small GTPases that cycle between membrane GTP-bound and soluble GDP-bound forms, assist this vesicular budding. Proteins that cannot fold correctly or assemble into oligomers are recognized by the ER quality control apparatus and eliminated before they get into the secretory pathway (Ellgaard and Helenius, 2003).

This system ensures that only fully-folded/assembled protein complexes are delivered to their final destination, thereby preventing any deleterious effect. Studies conducted with mutated, misfolded or incompletely assembled proteins revealed that recognition by the quality control apparatus operates on a structural rather than functional basis (Romisch, 2005). ER quality control operates on several and partially overlapping pathways. These include retrieving misfolded proteins from the Golgi by
Figure 10. **Secretory pathway organelles within a eukaryotic cell.** Secretory proteins are translocated into the rough ER and properly folded by various ER resident chaperones and folding enzymes. The correctly folded proteins are secreted towards the Golgi apparatus through COPII vesicles (black arrow) and eventually to their final destination (black arrow). Terminally misfolded proteins elicit a quality control response that operates through multiple but partially overlapping mechanisms. These include retrieval from the Golgi by COPI vesicles (red arrow), or diversion towards the lysosomes/vacuoles for degradation (green arrow). Misfolded protein overload also can also lead to ERAD and the UPR response (blue arrows).

COPI vesicles (Spang et al., 1998), the unfolded protein response (UPR) (Hampton, 2000), autophagy (Levine and Klionsky, 2004), and the endoplasmic reticulum-associated degradation pathway (ERAD) (Fig. 10) (Meusser et al., 2005).

**COPI retrieval from the Golgi-complex**

ER resident proteins are retrieved from the Golgi through COPI vesicles (Rothman and Orci, 1992; Spang et al., 2001). Some misfolded proteins can also escape the ER quality control apparatus, largely under stress conditions when proteins cannot be
retained in the ER or the ER apparatus does not identify the folding defect. These proteins are recognized in the early Golgi compartments and transported either back to the ER for cytosolic degradation or to the vacuole/lysosome for degradation (Appenzeller-Herzog et al., 2005).

**Unfolded protein response (UPR)**

The Unfolded Protein Response (UPR) is initiated when there are a large number of unfolded/misfolded proteins present in the ER lumen. UPR alleviates stress by transcriptional regulation of the synthesis of ER chaperones and folding enzymes synthesis and/or inhibition of secretory protein synthesis (Zhang and Kaufman, 2006).

The effects of the UPR increase the capacity of the cell to cope with the unfolded proteins. If UPR signals are present for a prolonged time, the cell ultimately kills itself by apoptosis (Fig. 12) (Di Sano et al., 2006). The UPR signaling components were first identified in *Saccharomyces cerevisiae* (Cox et al., 1993; Mori et al., 1993) (Fig. 11). UPR is less complex in budding yeast than in mammalian cells.

There are three independent UPR pathways present in metazoans (Ire1, ATF6, and PERK) (Bernales et al., 2006) (Fig. 12). The Ire1 protein has two isoforms: Ireα and Ireβ in mammalian cells that are expressed cell-specifically (Fig. 11). The PERK protein is similar to Ire1 in its lumenal unfolded protein sensing domain, but it cytosolic domain contains an eIF2α kinase domain (Fig. 12ii) (Bertolotti and Ron, 2001; Zhang et al., 2005). The lumenal domains of both Ire1 and PERK dimerize when there is a significant amount of unfolded protein present in the ER lumen, and in turn their cytosolic domains go through auto-phosphorylation (Liu and Kaufman, 2003).
Figure 11. Mechanism of Ire1-mediated mRNA splicing. Unfolded proteins on the lumenal side activate Ire1, resulting in its dimerization and autophosphorylation of the cytosolic kinase (K) domain of Ire1. This phosphorylation event in turn causes the activation of the endoribonuclease domain (R). The ribosomes on the HAC1 mRNA are stalled due to base pairing between the 5'-UTR and an intron. The R-domain removes this intron, and the tRNA ligase splices the exons so the stalled ribosomes can continue the translation efficiently. The product, transcription factor Hac1, enters the nucleus to activate the target genes. This figure was adapted from (Bernales et al., 2006).
Figure 12. The metazoan unfolded protein response (UPR) consists of three branches. There are three different ER stress transducers: ATF6 (i), IRE1α and β (ii) and PERK (iii). These proteins recognize the unfolded protein content present in the ER lumen and convey this through the membrane to trigger transcription factors or initiate translational control. In mammalian cells, ATF6 also induces XBP1 mRNA expression. All the transcription factors have a combined effect on UPR target genes to cause the protein folding capacity of the cell to enlarge and consequently aid in restoring homeostasis. PERK also contributes to the decrease in overall translation in cells, thus causing less protein transport into the ER. Unsuccessful restoration of ER homeostasis eventually results in apoptosis. This figure was adapted from (Bernales et al., 2006).

One underlying mechanism consists of an ER membrane RNase (Ire1p) that transmits a signal through the ER membrane when the concentration of unfolded proteins in the lumen gets too high, and this signal activates a transcriptional regulator, Hac1, which in turn controls the synthesis of an increasing amount of ER chaperones (Cox and Walter, 1996; Mori et al., 1996) (Fig.11). The Ire1 UPR pathway is present also in mammalian cells and is integrated into some multiple, parallel, and cross-wired conduits (Leber et al., 2004; Patil et al., 2004). Ire1/Hac1 transcription factors control
over 400 different genes, and in addition to upregulating ER chaperones, the UPR also codes for increased phospholipid synthesis, ERAD, and vesicular secretion (Travers et al., 2000). The recent crystal structure of Ire1 showed homology to the peptide binding groove of MHC-I chains, implying that the lumenal domain of Ire1 may bind to unfolded proteins in the ER lumen (Credle et al., 2005). Earlier, negative regulation by BiP was proposed, but refuting this hypothesis, these lumenal BiP binding sites have been identified on Ire1, and the deletion of this region did not have any affect UPR (Oikawa et al., 2005).

Another member of the UPR response family in mammalian cells is the ATF6 protein (Fig. 12i). ATF6 is also constitutively expressed throughout the cell (Shen et al., 2005; Ye et al., 2000). ATF6 belongs to the cAMP regulatory element-binding protein family. ATF6 becomes cleaved by the S1P and S2P proteases when UPR is induced. S1P and S2P were first identified as proteases activated by cholesterol starvation. After ATF6 is cleaved due to ER stress and released from the ER membrane, it travels to the nucleus to activate its target genes (Zhang and Kaufman, 2006). The third component of the mammalian UPR response is PERK (PKR-like ER kinase), an ER type I transmembrane protein (Fig. 12iii). Its major job is to inhibit translation in the cytosol by phosphorylating eIF2α, which in turn interferes with the formation of 43S translation initiation complex formation (Harding et al., 2000).

If the cell is subjected to extensive ER stress, all three branches of UPR trigger apoptosis, which is accompanied by a large Ca²⁺ release from the ER. Specifically, preapototic genes, such as CHOP are upregulated by both the PERK and ATF6
pathways (Yoneda et al., 2001). CHOP decreases Bcl-2 levels and thereby sets off the release of mitochondrial cytochrome c and the apoptosis inducing signal cascade (Urano et al., 2000). Simultaneously Ire1 activates the JNK cascade via TRAF2 protein, and the ER resident caspase-12 and 4. Additionally, Ca^{2+} release has a major role in calpain activation, which prompts the protease cascade. It is unknown presently how the unfolded proteins in the ER can trigger Ca^{2+} release (Schroder and Kaufman, 2006).

**Autophagy**

There are conditions in vivo under which all the quality control pathways fail and the misassembled proteins form large aggregates. Autophagy involves the recognition and engulfing these species (target proteins or organelles) by autophagosome vesicles (Bernales et al., 2007). These membrane vesicles then fuse together with the lysosome to release their contents for degradation via the lysosomal enzymes (Levine and Klionsky, 2004). Several diseases (e.g. Huntington’s, Parkinson’s) are caused by the accumulation of protein aggregates in intracellular compartments. Experiments showed that these large protein aggregates have less deleterious effect then smaller aggregates (Yorimitsu and Klionsky, 2007). In some cases, these aggregates can be found ubiquitinated, suggesting that the ubiquitin-proteasome system plays a role in the cleaning out process, even though these large misfolded proteins complexes may present a problem for the proteasome (Bennett et al., 2005). Ubiquitination of these large complexes might have a role in autophagy. For example, autophagy eliminates A1PiZ when it is assembled into aggregates (Fig. 4) (Kruse et al., 2006).
Endoplasmic reticulum-associated degradation (ERAD)

The ERAD pathway, one aspect of ER quality control, ensures irreversibly misfolded or unassembled proteins are eliminated before secretion. The first evidence that misfolded proteins were retained in the early secretory pathway and degraded came from studies with misfolded T-cell receptor (TCRα) and mutant cystic fibrosis conductance regulator (CFTR) (Cheng et al., 1990; Yu and Kopito, 1999). This intracellular degradation was once thought to occur in the ER lumen or in a pre-Golgi compartment. At the same time it was hard to comprehend how such an aggressive proteolytic apparatus can be present in the ER lumen, where there are a large number of unfolded proteins present. Experiments during the last decade showed that ERAD substrates were transported back to the cytosol for degradation (Fig. 4) (Bonifacino et al., 1989; Meusser et al., 2005).

Even though the underlying mechanisms of ERAD are not completely understood, the current experimental approaches have elucidated a series of events that lead to degradation. First, folded proteins must be distinguished from folding intermediates and misfolded proteins, although it is unclear how the cell recognizes the myriad of conformational states and classifies them. It is plausible that the unfolded proteins stay in the ER by default, while properly-folded proteins are sorted into COPII-coated vesicles for transport into the Golgi apparatus. But this type of selection would need specific receptors that recognize folded conformations or glycosylation states (discussed later). Some membrane proteins contain a dibasic motif (transmembrane domains of T-cell receptor and μ heavy chain of IgM, or cytosolic domain of IgE, ATP sensitive K⁺
channel, or CFTR Δ508) that serves a signal for retention in the ER. Hence, masking this dibasic signal upon proper assembly may induce proper secretion from the ER (Stevens and Argon, 1999).

Many human diseases [(Creutzfeldt-Jakob disease (CJD)], Scrapie, bovine spongiform encephalopathy (BSE), or Parkinson and Alzheimer) are caused by protein aggregates (Boelens et al., 2007; Rutishauser and Spiess, 2002; Yoshida, 2007). Some others, such as cystic fibrosis and lung emphysema, result from the early degradation of proteins (CFTR and α-1 antitrypsin respectively) (Fig. 13) (Jensen et al., 1995; Qu et al., 1996; Rutishauser and Spiess, 2002; Ward et al., 1995). Yeast genetics has been a powerful tool in elucidating the role of various chaperones and folding enzymes in ERAD, although these findings can not be applied entirely to the more complicated mammalian systems (Brodsky and McCracken, 1997; Plemper et al., 1997). Numerous experiments in yeast cells involved a soluble ERAD substrate, a mutant version of carboxypeptidase Y (CPY*). This protein has an active site mutation (Gly to Arg) that results in an altered, trypsin-sensitive structure (Finger et al., 1993). Yeast ERGIC (sec18, ufe1, sed5, erv29) and COPII (sec12 and sec23) mutants showed decreased CPY* degradation, which suggested that the ER to Golgi transport is necessary for the ERAD of this soluble protein (Caldwell et al., 2001; Taxis et al., 2002; Vashist et al., 2001; Vashist and Ng, 2004).

Experiments revealed the presence of some sub-compartments in the ER that are formed specifically for ERAD substrates that travel from the Golgi via retrograde
**ERAD substrates in yeast:**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pro-α-factor</td>
<td>Peptide hormone</td>
</tr>
<tr>
<td>A1PIZ</td>
<td>Human α1-protease inhibitor</td>
</tr>
<tr>
<td>CPY*</td>
<td>Carboxypeptidase Y</td>
</tr>
<tr>
<td>Fur4p</td>
<td>Uricil Permease</td>
</tr>
<tr>
<td>Hmg2p</td>
<td>HMG-CoA reductase</td>
</tr>
<tr>
<td>Pdr5p*</td>
<td>ABC (ATP binding cassette) transporter</td>
</tr>
<tr>
<td>Sec61p</td>
<td>Translocon component</td>
</tr>
<tr>
<td>Ste6p</td>
<td>α-Factor transporter</td>
</tr>
<tr>
<td>Vph1p</td>
<td>V-ATPase subunit</td>
</tr>
</tbody>
</table>

**ERAD substrate Associated disease:**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>a1-protease inhibitor (A1PIZ)</td>
<td>Emphysema and liver disease</td>
</tr>
<tr>
<td>Apolipoprotein B100 (apoB100)</td>
<td>Lung emphysema, liver disease</td>
</tr>
<tr>
<td>Aquaporin-2 (AQP2)</td>
<td>Nephrogenic diabetes insipidus</td>
</tr>
<tr>
<td>Cystic fibrosis transmembrane regulator (ΔF508 CFTR)</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>HMG-CoA reductase</td>
<td>Heart disease, Cholesterolemia</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>Wilson disease</td>
</tr>
<tr>
<td>LDL receptor class II mutants</td>
<td>Diabetes mellitus, Type A insulin resistance</td>
</tr>
<tr>
<td>Lyosomal α-galactosidase A</td>
<td>Familial hypercholesterolemia</td>
</tr>
<tr>
<td>Prion protein (Prp30)</td>
<td>Fabry disease</td>
</tr>
<tr>
<td>Thyroglobulin (Tg)</td>
<td>Neurodegenerative disease (Creutzfeldt-Jacob disease)</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>congenital hypothyroid goiter</td>
</tr>
<tr>
<td>Wilson protein</td>
<td>Amelanotic melanomas</td>
</tr>
<tr>
<td>Von Willebrand factor</td>
<td>Wilson disease</td>
</tr>
<tr>
<td>Beta-hexosaminidase</td>
<td>Von Willebrand’s disease</td>
</tr>
<tr>
<td>CD4</td>
<td>Tay-Sachs disease</td>
</tr>
<tr>
<td>Collagen</td>
<td>AIDS</td>
</tr>
<tr>
<td>Connexin</td>
<td>Osteogenesis imperfecta</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Charcot-Marie-tooth disease</td>
</tr>
<tr>
<td>MHC class I</td>
<td>Familial hypofibrinogenemi</td>
</tr>
<tr>
<td></td>
<td>HEF Infantile (CMV) hepatitis</td>
</tr>
</tbody>
</table>

**Opportunistic uses of ERAD:**

- Viruses escape immunodetection via ERAD
- MHC class I heavy chain
- HMCV US2 and US11 mediated degradation
- CD4 receptor
- HIV-1 Vpu induced degradation
- Bacterial and plant toxins invade the host cell via ERAD
- Cholera toxin A1 chain (CTA1)
- Pertussis toxin
- Ricin A chain (RTA)
- Shiga toxin

**Other ERAD substrates:**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribophorin I (RI332)</td>
<td>Subunit of OST</td>
</tr>
<tr>
<td>ATP-sensitive K⁺ channel</td>
<td>Degraded when unassembled</td>
</tr>
<tr>
<td>T-cell receptor α-chain</td>
<td>Degraded due to unfolding</td>
</tr>
<tr>
<td>Human α-opioid receptor</td>
<td>Untrimmed glycans</td>
</tr>
<tr>
<td>Human IgE-receptor</td>
<td></td>
</tr>
<tr>
<td>Cell surface IgM υ-chain</td>
<td>Degraded without light chain</td>
</tr>
</tbody>
</table>

Figure 13. Some ERAD substrates and their relevance in the cell. Some ERAD substrates have medical significance, while others are used by pathogens to invade the host system. This figure was adapted from (Dalbey and von Heijne, 2002).
movement (Vashist et al., 2001). However, yeast genetics provide only indirect evidence, because these mutants also disturb ER homeostasis and mislocalize several ER resident proteins (Nishikawa et al., 1994; Prinz et al., 2000). Fluorescence and electron microscopy data showed that the Ste6p* localized in the ER to proliferated ER membranes, which was later named ER-associated compartments (ERAC) (Huyer et al., 2004a). ER chaperones were also present in this ER network, which suggested that there is not any obstruction between the ER and ERAC. This group proposed that these misfolded membrane proteins may be stored in this compartment until ERAD can take place (Huyer et al., 2004b).

Retrotranslocation of CPY* in yeast requires Kar2p and Der1p (Knop et al., 1996) several J-domain proteins (Sec63p, Scj1p and Jem1p) (Nishikawa et al., 2001), and also some ubiquitination factors: Ubc7p, Ubc6p, (Hiller et al., 1996), Ubc1p (Friedlander et al., 2000), Hrd1p (Der3p) (Hampton et al., 1996; Plemper et al., 1998), and Hrd3p (Gardner et al., 2000). CPY* also contains four glycan moieties, so its degradation also depends on carbohydrate-sensitive ERAD components, and Htm1p (EDEM) (Hosokawa et al., 2003; Hosokawa et al., 2001).

Several bacterial and plant toxins exploit the ERAD machinery. A/B toxins (e.g. Pertussis and cholera toxins) translocate their toxic proteins into the ER of the infected organism (Tsai and Rapoport, 2002; Worthington and Carbonetti, 2007), while others (ricin) use receptor-mediated endocytosis to reach the ER and retrotranslocate back to
the cytosol (Lord et al., 2003). These toxins usually have a low lysine concentration to avoid degradation (Lord and Roberts, 1998).

Proteins without glycan moieties have so far received less attention. It was suggested that the lumenal proteins involved in ERAD substrate selectivity include Hsp70 molecular chaperones that detect and associate with misfolded polypeptides. Specifically, the ER lumenal Hsp70 (BiP in mammals and Kar2p in yeast) and two Hsp40 co-chaperones assist ERAD in yeast. Hsp70 presumably functions in ERAD to maintain the misfolded proteins in a non-aggregated state. Kar2p (yeast BiP) is required for the degradation of the mutant pro-α-factor (Δgpαf) and CPY* in yeast, but not of membrane proteins (Brodsky, 1996; Brodsky, 2005; Brodsky et al., 1993; Brodsky and McCracken, 1997; Brodsky and McCracken, 1999; Brodsky et al., 1999; Plemper et al., 1997; Plemper et al., 1998; Plemper and Wolf, 1999).

One proposed role for BiP is to transport the aggregation prone proteins directly or indirectly to another ER resident protein that might serve as the actual retrotranslocation channel (Nishikawa et al., 2005; Nishikawa et al., 2001). BiP also seals the translocon from the lumenal side, which positions it in a close proximity to the translocon. If the translocon also serves as a retrotranslocon, this gate-keeping function of BiP also could explain its proposed role in ERAD substrate targeting (Haigh and Johnson, 2002; Hamman et al., 1998).

PDI (protein disulfide isomerase), a thiol-disulphide oxidoreductase has also been suggested to play a role in ERAD. While CPY* and Igµ chain (Fagioli et al., 2001) degradation was understandably inhibited in a PDI-deficient yeast mutant strain due to
their disulfide bonds. A cysteine free protein, Δgpαf, was astonishingly found to require PDI in yeast (Gillece et al., 1999; Werner et al., 1996). This finding suggests that PDI can also act as a molecular chaperone. PDI also has been described as a redox-driven chaperone in a mammalian cell line required for unfolding the A1 chain of the cholera toxin before targeting it to the ER membrane (Tsai and Rapoport, 2002; Tsai et al., 2001; Tsai et al., 2002). This group proposed that PDI binds to the substrate in a reduced state, forms disulfide bonds with the substrate, and travels with the substrate to a membrane complex containing the oxidase Ero1. Ero1 would then oxidize PDI, which in turn would release its substrate, and perhaps direct it to a retrotranslocation component located in the ER membrane. The assertion that PDI is a redox driven chaperone was later questioned by experiments that proved that substrate binding and release are not driven by the oxidation state of PDI (Lumb and Bulleid, 2002). Another thioredoxin, Eps1, the ER membrane protein version of PDI, is required for degradation of the yeast membrane ATPase (Pma1p) (Wang and Chang, 1999). The CNX/CRT-associated Erp57 might have a distinct role in the glycoprotein quality control (Farmery et al., 2000; Oliver et al., 1999; Wang et al., 2005).

The most characterized ERAD process to date involves glycoprotein degradation. Although this process is becoming increasingly well understood, there are still many unknowns (Helenius and Aebi, 2004; Trombetta, 2003). When N-glycosylation is inhibited due to mutations or inhibitors (tunicamycin), proteins that are normally glycosylated, are subjected to aggregation and/or misfolding (Mancini et al., 2003; Obinata et al., 1996), perhaps because the negatively-charged hydrophilic glycan
Figure 14. Processing and quality control of N-glycosylated proteins in the ER. (1) The core glycan is assembled partly in the cytosol and flipped to the lumenal side in an ATP-dependent manner. In the lumen the glycan moiety is fully assembled and loaded onto the oligosaccharyl transferase (OST) complex. (2) The N-glycans \(\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\) are co-translationally conjugated to the incoming nascent chain by the OST complex. (3) The carbohydrate moiety is then trimmed by glucosidase I and II (GI and GII) to a mono-glucosylated state and enters the calnexin (CNX) or calreticulin (CRT) folding cycle (while this process can occur co-translationally, it is not shown for simplicity)(4). If the substrate has thiol groups, Erp57 may assist the disulfide bond formation (4). (5) Trimming of the last glucose residue by GII promotes the release of substrate from CNX/CRT. (6) If the CNX/CRT released substrate is not properly folded, it may be re-glucosylated to the mono-glycosylated form by UDP-glucose: glycoprotein glucosyltransferase (UGGT). This causes the substrate to bind again to CNX/CRT and undergo another series of folding attempts. If the protein eventually reached its native conformation it becomes trimmed by ER mannosidases I and/or II (Man I and Man II) to obtain a high-mannose glycan conformation (M-N) that can bind to ERGIC-53 (7) and packed into COPII vesicles for secretions towards the Golgi (8). The misfolded glycoproteins that enter in and out of the CNX/CRT folding cycle ultimately become trimmed by ER mannosidase I (Man I) to a Man\textsubscript{8}GlcNAc\textsubscript{2} and either (9) bind to EDEM (which can be soluble or membrane bound) (10) or interact with various ER-resident chaperones/folding enzymes (Yos9p, BiP, PDI etc.)(10). The substrate may be retained in the ER for folding and rescue attempts by these chaperones (10), but if the protein is still misfolded, then it is eventually relayed to the retrotranslocation apparatus either directly (11) or indirectly (through EDEM). (11) EDEM-bound substrates are targeted for retrotranslocation to the cytosol. (12) The ERAD substrate traverses the ER membrane. The AAA-ATPase P97 and/or 19 RP might drive the dislocation of the possibly unfolded chain via an unknown retrotranslocon. In the cytosol, various E3, deglycosylation (Png1), deubiquitination enzymes interact with the glycoprotein before the proteasomal degradation. This figure was adapted from (Hebert et al., 2005).
facilitates the proper folding and/or prevent premature aggregation of incoming nascent chain. Hence, it is not surprising that proteins involved in N-glycosylation also participate in ERAD. These include CNX, CRT, UGGT and ManI and II (the N-glycosylation machinery as discussed above) (Fig. 14). Glycan moieties on misfolded proteins must retain a glucose residue to interact with CNX/CRT and undergo the re- and deglucosylation in order to fold properly (Ruddock and Molinari, 2006). UGGT has a major role in the CNX/CRT cycle and preferentially interacts with misfolded proteins (Guerin and Parodi, 2003).

The most important job in glycoprotein quality control has been attributed to $\alpha 1,2$-mannosidases (Suzuki et al., 2006). The first observations that mannose trimming plays an important role in the degradation of misfolded proteins originated from inhibition studies (Helenius, 1994; Molinari et al., 2002; Su et al., 1993; Wu et al., 2003). When specific $\alpha 1,2$-mannosidase inhibitors, Kif or dMNJ were used to inhibit mannose-trimming activity or when yeast ManI was mutated, a delay in misfolded protein disposal was observed (Kawar and Jarvis, 2001; Kawasaki et al., 2007; Kitzmuller et al., 2003; Lu et al., 2006). When ManI was overexpressed, the misfolded proteins were eliminated faster. These findings led to the theory of “mannose-timer” (Helenius and Aebi, 2001; Liu et al., 1999). Removal of mannose residues would decrease the affinity of UGGT for the folding intermediate, which in turn would not enter the CNX/CRT cycle and/or would fold with a reduced speed, thereby making it possible for the ERAD machinery to recognize and eliminate this misfolded protein.
*Saccharomyces cerevisiae* does not contain any UGGT or CRT; even its CNX homologue (Cne1p) is very different from the mammalian one. Although the mannose trimming mechanism is present in *S. cerevisiae*, it is a much simpler version of the mammalian counterpart, which is evolutionarily more developed and complex. Recent studies demonstrated that mannose trimming can proceed up to GlcMan$_{5,6}$GlcNAc$_2$ (Foulquier et al., 2004; Frenkel et al., 2003; Hosokawa et al., 2003; Hosokawa et al., 2001; Kitzmuller et al., 2003).

The lectin, EDEM, is important for capturing the substrate after mannose removal. EDEMs possess the glycan-binding pocket, but with no apparent hydrolytic activities (Molinari et al., 2003), although the latter is debated (see above) (Olivari et al., 2006). Even though the role of EDEM is unknown, overexpression of either EDEM 1 or 3 resulted in the elimination of misfolded N-glycans at a faster rate. When the EDEM gene was disrupted, the retrotranslocation rate decreased (Hirao et al., 2006; Hosokawa et al., 2003; Hosokawa et al., 2001; Mast et al., 2005; Molinari et al., 2003; Movsichoff et al., 2005; Oda et al., 2003; Oda et al., 2006; Olivari et al., 2006; Olivari et al., 2005). Interaction between EDEM and the misfolded N-glycans has been shown by co-immunoprecipitation (Oda et al., 2003). Experiments also demonstrated that CNX transfers an ERAD substrate to EDEM (Oda et al., 2003). Mammalian Derlin-2 and 3 has been discovered to interact with EDEM-1, which might form the putative retrotranslocation channel (Figs. 15Av. and Biv.) (Oda et al., 2006). This study proposed that EDEM-1 might transfer the glycoprotein target to the dislocation site on the membrane and eventually to p97 (Oda et al., 2006). EDEM might prevent
Figure 15. Different ERAD complexes aid distinct ERAD substrates. Some of the most important ERAD components, but not all of them identified to date are shown on panels (A) and (B). (A) The ERAD-C pathway. ERAD substrate with a cytosolic misfolded region (yellow star) may rely on a different dislocation pathway than those substrates with a lumenal or transmembrane abnormality (B). ERAD-C substrates may require an ubiquitin ligase, Doa10p in complex with Ubx2p. Ubx2p can recruit the Cdc48/p97 complex via its UBA domain (Neuber et al., 2005) (ii). The putative retrotranslocon (either the Sec61 channel (i) or Der1 (iii)) may associate with the E3 complex. Der1 might also form a complex with VIMP, (a single-spanning membrane protein that may bind Cdc48/p97) (Ye et al., 2004). The Der1/VIMP complex alone or in association with Der2-3 (iv) (Oda et al., 2006), or these components in different combinations, constitutes the putative retrotranslocon. There is evidence that Sec61 may either be the part of this channel or associates with some of these membrane components (Plemper et al., 1997). The nature of the retrotranslocon is still largely unknown. If the ERAD substrate contains a cytosolic lesion, it requires soluble cytosolic factors for its extraction. These proteins include CHIP (an E3 ligase) (Younger et al., 2006), cytosolic Hsp70 (Meacham et al., 2001), or Hsp104 (Taxis et al., 2003). Ubiquitin-conjugation enzymes Ubc7p and Ubc1p (not shown) may also play a role. The membrane extraction of an ERAD-M substrate may be facilitated by either the Cdc48/p97 complex (Ye et al., 2003), the 19S RP (Wahlman et al., 2007) or a combination of both. (B) ERAD-L and ERAD-M substrates with the misfolded region in either the lumen or in the membrane, respectively. It has been proposed that either the Sec61 channel (i) or the Hrd1 E3 complex (ii) or Der1 (iii) in combination with (i) or (ii) or (iv) form the retrotranslocon pore. How this ERAD substrate crosses the membrane is still largely unknown. On the luminal side they interact with soluble luminal chaperones like PDI/BiP and/or the N-glycosylation machinery. On the cytosolic side, they might be extracted by Cdc48/p97 or 19S RP, which was reported to interact with the translocon (Ng et al., 2007). Usa1 is thought to recruit the AAA complex and may connect Der1 to the Hrd1 complex (Carvalho et al., 2006). Hrd3 (an E2 enzyme) has a large luminal domain, which can gather the ERAD-L substrates on the luminal side for retrotranslocation (Gardner et al., 2000; Mueller et al., 2006; Plemper et al., 1999a). There could be still many unidentified components in both (A) and (B).
premature aggregation and disulfide bond formation. One research group claimed that chaperoning activity was not linked to the mannosidase-like activity, because EDEM mutants that were defective in their catalytic domain could still interact with the substrate similar to wild type EDEM (Olivari et al., 2006).

Yos9p, another lectin in the yeast ER lumen, has been reported by three different groups simultaneously to participate in a retrotranslocation associated process (Bhamidipati et al., 2005; Kim et al., 2005; Szathmary et al., 2005). Co-immunoprecipitation established a direct interaction between Yos9p and a misfolded ERAD glycoprotein substrate that contains either GlcMan₅GlcNAc₂ or GlcMan₄GlcNAc₂. These three independent studies disagreed on several issues, primarily the contribution of EDEM-1 to the Yos9p-mediated interactions and the deciding factor in substrate recognition. These groups all agreed on the important role of Yos9p in glycoprotein ERAD. Yos9p has a mammalian homologue, OS-9 (Carvalho et al., 2006).
An ERAD-L substrate must traverse the ER membrane to degrade in the cytosol. The soluble ERAD substrates must exit the ER, presumably through an aqueous pore-complex; similar to the translocon which promotes the forward translocation. It was proposed, based on data with yeast translocon mutants, that both soluble and membrane proteins are exported from the ER through the Sec61 translocon (Ali et al., 2005; Pilon et al., 1997; Plemper et al., 1999b; Plemper et al., 1999a; Sommer and Wolf, 1997) (Figs. 15Ai and Bi). It is conceivable that the translocon has several operational modes (Johnson and Haigh, 2000), and several translocon associated proteins facilitate the putative switch between retro- or forward translocation. However, the data are not completely convincing, because it was not clear in the studies done to date whether the substrate molecules were on their way out to the cytosol or into the lumen. The human cytomegalovirus (HCMV) produces a membrane-anchored protein, called US2. US2 causes the class I major histocompatibility complex (MHC) to retrotranslocate from the ER to the cytosol, and US2 was found in association with the Sec61 translocon (Wiertz et al., 1996a; Wiertz et al., 1996b).

It is unclear whether the same route is utilized by all glycosylated ERAD substrates and also how these substrates are targeted to the retrotranslocation site (Fig 15). It has also not been determined to date whether ERAD substrates exit the ER in an unfolded state, and if so, whether one end moves through the membrane preferentially. Newly-synthesized proteins enter the ER lumen in an unfolded state, so when they may exit through the ER membrane they also need to be unfolded. Consistent with this idea, cholera toxin is unfolded by PDI before dislocation from the ER (Tsai and Rapoport,
2002; Tsai et al., 2001; Tsai et al., 2002). Yet some fully-folded domains (DHFR and GFP) conjugated to an ERAD substrate also retrotranslocated, implying that unfolding is not necessary for retrotranslocation (Fiebiger et al., 2004; Fiebiger et al., 2002; Tirosh et al., 2003; Tirosh et al., 2005). If folded proteins are retrotranslocated, the retrotranslocation pore has to be large in diameter, because DHFR and GFP have diameters of 40 and 24 Å diameters respectively. Glycosylated substrates also return to the cytosol with a complete set of glycosylation moieties where they are removed by deglycosylation enzymes (Petaja-Repo et al., 2001).

The crystal structure of a Sec61 homologue (archaeal SecYEG) indicated that the translocon has a 10 –12 Å diameter, which would be inconsistent with its proposed retrotranslocation function (Osborne and Rapoport, 2007; van den Berg et al., 2004). But the crystal structure is unable to provide data on the flexibility and dynamic nature of the translocon (Hamman et al., 1997; Johnson and van Waes, 1999; Wirth et al., 2003). In contrast, fluorescence quenching experiments indicated that a translocon engaged with the translating ribosome has a pore diameter of a 40-60-Å (Hamman et al., 1997).

It is also possible that another integral ER membrane protein with an unknown function may facilitate the export of ERAD substrates through the membrane (Hitt and Wolf, 2004; Knop et al., 1996) (Figs. 15Aiii and Bi). For example, it has been recently discovered that Derlin-1 (Der1 or Der1p in yeast) with four transmembrane domains in the ER membrane may have such role in ERAD (Ye et al., 2004). An interaction of Der1 with class I MHC upon US11-dependent degradation has been detected by co-immunoprecipitation, a finding that led to suggested that Der1 may be a part of the
retrotranslocation channel (Lilley and Ploegh, 2004), yet Der1p does not seem to be required for all the ERAD substrates in yeast (Taxis et al., 2003). Other likely candidates are some ubiquitin ligases (Hrd1p and Doa1p) that span the membrane several times (Fig. 15Ai and 15Bi). This would provide an opportunity to link retrotranslocation to ubiquitination at the same time (Deak and Wolf, 2001; Gardner et al., 2000; Kota et al., 2007; Kreft et al., 2006).

The next question is how polypeptides are released into the cytosol. Retrotranslocation probably requires a driving force to facilitate unidirectional movement of an ERAD substrate from the lumen. Most, but not all ERAD substrates are polyubiquitinated, and ubiquitin conjugation might serve as a 'ratchet', if polyubiquitination (discussed later) and export are coupled (Biederer et al., 1997; de Virgilio et al., 1998; Shamu et al., 2001). It has been proposed that cytosolic molecular chaperones might assist polypeptides in exiting the ER. During post-translational translocation into the ER and mitochondria, BiP and mitochondrial Hsp70, respectively, power the translocation of polypeptides. Hence, retrotranslocation may be similar to post-translational translocation, except in the opposite direction.

Experiments in the presence and absence of impaired 26S proteasomes showed that ERAD substrates with both integral membrane spanning and cytosolic regions had their cytoplasmic regions degraded more rapidly than the membrane spanning domains (Mayer et al., 1998; Walter et al., 2001). This finding suggested that the proteasomal 19S cap might play a role in the extraction of the integral membrane segments (Fig. 15). If true, the 19S cap of the 26S proteasome may power the ATP-dependent extraction of the
substrate while the 20S proteolytic subunit degrades the substrate (Hirsch and Ploegh, 2000). Using a yeast in vitro assay, it was shown that yeast cytosol can be substituted by purified yeast or mammalian proteasomes to fulfill the requirements for degradation of an ERAD substrate (Lee et al., 2004). When the proteasomal degradation activity was inhibited, most of the ERAD substrates can be located in the cytosol (Lee et al., 2004). These results suggest that extraction and degradation were uncoupled, and the substrate is retrotranslocated through the membrane by either another protein (e.g. Cdc48/p97; see below) or only the 19S cap of the proteasome (Jarosch et al., 2002; Johnston et al., 1998; Wiertz et al., 1996b).

Consistent with this model, the Cdc48 (p97)/Ufd1/Npl4 complex on the cytosolic side of the membrane may aid the retrotranslocation and extraction of some ubiquitinated ERAD substrates (Bays et al., 2001a; Bays et al., 2001b; Braun et al., 2002; Hitchcock et al., 2001; Jarosch et al., 2002; Rabinovich et al., 2002; Ye, 2006; Ye et al., 2001). The first evidence that Cdc48/p97 was involved in degradation came from studies that examined an unfolded substrate with an N-terminal ubiquitin conjugation (Ghislain et al., 1996).

Cdc48/p97 could also act after the ERAD substrate was extracted from the membrane, as was shown by work done with the Spt23p precursor protein proved this hypothesis (Hitchcock et al., 2003; Hitchcock et al., 2001; Rape et al., 2001). This protein is an integral membrane protein that forms a homodimer called p120 (120 kDa). It becomes cleaved upon activation to a membrane bound and a soluble fragment, p90 (90 kDa), and this proteolysis step takes place after ubiquitination of the p120. Since this
ERAD substrate accumulated on the cytoplasmic face when mutant yeast cells had defective Npl4 and Ufd1, p97/Npl4/Ufd1 appear to be involved (Bays et al., 2001b; Jarosch et al., 2002). These data implied that Cdc48/Npl4/Ufd1 removed the ubiquitinated p90 from the membrane-bound p120. A recent study also suggested that Cdc48/Npl4/Ufd1 might bind to a monomeric or very short ubiquitin chain on p90 (Richly et al., 2005). These short ubiquitin chains were then further elongated by Ufd2p and eventually passed on to Rad23p/Dsk2p from Cdc48/Npl4/Ufd1, which then delivered p90 to the proteasome (Chen and Madura, 2002; Funakoshi et al., 2002). This process was named the “escort pathway” that hands over the ERAD substrate to a set of proteins with affinities for different ubiquitin chains (Richly et al., 2005). Rad23p was also found to interact with a deglycosylation enzyme, Png1p (Fig.14) (Elsasser et al., 2004; Medicherla et al., 2004; Suzuki et al., 2001).

Cdc48 [p97 or VCP (valosin-containing protein) in mammals] belongs to the AAA-ATPase family. Both Sec18p and Cdc48 contain a SRH-motif (Second Region of Homology) and have duplications in the their ATP-binding domain (Frohlich et al., 1991; Lee and Wickner, 1992). X-ray structures elucidated the conformational changes that p97 goes through during the ATP hydrolysis cycle (DeLaBarre and Brunger, 2003; DeLaBarre and Brunger, 2005; DeLaBarre et al., 2006). This catalytic nucleotide-dependent structural change is responsible for its substrate unfolding activity (Dreveny et al., 2004; Yuan et al., 2004).

Cdc48 and the proteasome might interact directly with each other as well. Cdc48 preferentially works on proteins with a Ubx domain (similar to the structure of ubiquitin)
via its N-terminus (Decottignies et al., 2004; Schuberth and Buchberger, 2005; Schuberth et al., 2004). There have been seven Ubx-domain proteins identified in yeast to date (including Ubx1p/Shp1, the yeast p47 homologue). A Ubx-motif protein, specifically Ubx2p, is required for the degradation of several ERAD substrates such as CPY* (Neuber et al., 2005), and an artificial ubiquitin-containing protein by recruiting Cdc48/Ufd1p/Npl4p, Doa1p and Hrd1p (Fig. 15Aii and 16Bii). Ubx2p also interacts with Der1p and Dfm1p (a Der1p homologue in yeast), perhaps to form a putative retrotranslocation pore (Guerra et al., 2005; Lilley and Ploegh, 2004; Lilley and Ploegh, 2005; Schuberth and Buchberger, 2005; Ye et al., 2004). According to another group (Romisch, 2006), Cdc48 may bind directly to Der1p and causes Der1p to interact with the membrane-bound UBX domain of Ubx2p, while the UBA motif of Ubx2p contacts the Sec61 channel through ubiquitin ligases.

Ufd1p was discovered through screening for ubiquitinated mutants with decreased degradation (Johnson et al., 1995). The NMR structure showed separate binding sites for mono and polyubiquitin (Park et al., 2005). The N-terminal domain of Ufd1p is very similar to that of Cdc48, suggesting that Ufd1p also can bind to Ubx2p. The other co-chaperone of Cdc48, Npl4p, plays an important role in nuclear pore function and nucleus structure (DeHoratius and Silver, 1996). Npl4p mutant yeast strains showed no detectable impairment in cytosolic protein degradation, but some ERAD substrates accumulated in an ubiquitinated form in the cytosol, suggesting a role in retrotranslocation (Bays et al., 2001). Npl4p-Ufd1p heterodimer adaptors bind to Cdc48p via the Npl4p ubiquitin fold domain (UFD) (Hitchcock et al., 2001). This suggests that
Npl4p and Upd1p play a role in retrotranslocation after ERAD substrate ubiquitination, but before proteasome degradation (Bruderer et al., 2004; Hitchcock et al., 2001).

Another protein, Ufd2p, also binds to Cdc48p, but this interaction is inhibited by the membrane bound ubiquitin-ligase Doa1p (also called Ufd3p), which competes for the same binding site (Ghislain et al., 1996; Rumpf and Jentsch, 2006). A deubiquitination protein in yeast, Otu1p also utilizes the same docking site on Cdc48 (Rumpf and Jentsch, 2006). These interesting findings suggested that Cdc48 acts in the ubiquitination pathway as a core regulator, and its preference for different ubiquitin interacting proteins/adaptors determines the fate of its substrate. Unfortunately, currently there are no data available that would show whether specific chaperones, polyubiquitination, or the proteasome were itself aid retrotranslocation for ubiquitinated substrates.

Another uncertainty is whether the retrotranslocated proteins are degraded exclusively by 26S proteasome in the cytosol. Although the 26S proteasome is a major player in ERAD, alternative pathways are present in mammalian cells and can play an important role in the disposal of both glycoproteins and non-glycoproteins. It has been demonstrated that at least two ERAD substrates utilized both a proteasome-dependent and a proteasome-independent degradation pathways simultaneously (CPY* and A1PiZ in mammalian cells) (Brodsky et al., 1999; Kruse et al., 2006; Mancini et al., 2003; Marcus and Perlmutter, 2000; McCracken et al., 1996; Palmer et al., 2003; Qu et al., 1996; Teckman et al., 2001; Teckman and Perlmutter, 1996). RNA interference experiments identified the protease of the latter pathway as insulin degrading enzyme (IDE), which belongs to the M16 metalloprotease family (Schmitz and Herzog, 2004;
Schmitz et al., 2004). Similarly, experiments with ApoB revealed that proteasomes were responsible for only part of its degradation (Pariyarath et al., 2001).

It is likely that individual ERAD substrates will exhibit differential requirements for degradation since no two ERAD substrates to date were found to have identical requirements for their degradation. This is not surprising since ERAD substrates are either soluble in the lumen or integrated in the ER membrane (Fig. 15) where their domains are located in the cytosol, lumen, or ER membrane. As a result, a single pathway is perhaps inadequate to accommodate all substrates.

Experiments with the single-spanning \(\alpha\)-subunit of the T-cell receptor (TCR\(\alpha\)) membrane protein showed that the retrotranslocation of this protein is initiated from the lumenal side (Yu and Kopito, 1999), and its degradation depends on the Hrd1p ubiquitin ligase (Kikkert et al., 2004). This study also suggested that the degradation of both lumenal and membrane proteins depends on lumenal factors. Additionally, class I MHC degradation seems to be independent of cytoplasmic lysine residues that could be ubiquitinated (Furman et al., 2003; Shamu et al., 1999).

It has been proposed (Vashist et al., 2001) that most soluble ERAD substrates require lumenal, but not cytoplasmic, chaperones, while the degradation of most ER membrane proteins involves cytoplasmic, but not lumenal chaperones. A membrane protein, Ste6p was examined to identify what factors were involved in its retrotranslocation. The two halves of this protein each contain six transmembrane segments and a large cytosolic domain. Ste6p is rapidly degraded if this protein lacks its last 52 residues (Ste6p*) (Loayza et al., 1998). Ste6p* requires only cytosolic chaperones (Huyer et al., 2004a).
The cytosolic Hsp70 chaperone, Ssa1p, and its two co-chaperones (Ydj1p and Hlj1p) are involved, but not the luminal Kar2p. Ydj1p is attached to the ER membrane by prenylation, while Hlj1p is a signal-anchor membrane protein. Its degradation also depends on Doa10p ubiquitin ligase that associates normally with Ubc6p and Ubc7p, and directly or indirectly to Cue1p (Swanson et al., 2001). It was also shown that Hrd1p contributes to Ste6p* degradation, and not through the yeast Sec61 channel, but a homologue (Ssh1p) (Wittke et al., 2002). Another ER membrane protein that contains misfolded cytosolic domains is the mutant plasma membrane protein ATPase [(Pma1p (D378N)], and its degradation requires a disulfide-isomerase (Eps1p) (Wang and Chang, 1999; Wang and Chang, 2003).

Interestingly, when membrane proteins contain a misfolded luminal region, they require luminal chaperones (Fig. 15B). For example, the misfolded ABC multidrug transporter protein (Pdr5p*) (Plempner et al., 1998) needs Htm1p (EDEM) (Gnann et al., 2004; Jakob et al., 2001; Plempner et al., 1998) and Der1p, but not Kar2p. Studies with CPY* have taken this finding further, by fusing CPY* on the luminal side through a Pdr5p transmembrane segment to cytoplasmic GFP the obtain CTG* (Taxis et al., 2003). Degradation of this chimera depends on the proteasome and cytosolic Dsk2p, Rad23p, and Hsp70 with its co-chaperones (Hlj1p, Cwc23p, and Jid1p), but is independent of Der1p and Kar2p. The authors proposed that the misfolded luminal domain might signal through the GFP domain to recruit cytoplasmic chaperones.

Another protein chimera that has been constructed is signaling protein Wsc1p, with a single transmembrane segment fused to a KHN domain in place of the Wsc1p, N-
domain (Vashist et al., 2001) (KHN is a Kar2 signal peptide fused to simian virus5 HA-Neuraminidase ectodomain). This fusion protein, KWW, has a misfolded lumenal domain, and its degradation depends on Cue1p, Htm1p, Der1p, Hrd1p, and on ER-Golgi trafficking. When a different chimera (KSS) contained misfolded domains on both sides of the ER membrane, (Ste6p* in the cytosol and KHN in the lumen), the degradation required all of the components were necessary for degradation of Ste6p* protein (Huyer et al., 2004b). Thus, the use of modular substrates has elucidated some important features of the degradation of membrane proteins (Huyer et al., 2004a; Huyer et al., 2004b; Schulze et al., 2005; Taxis et al., 2003).

Another group used a systematic approach in yeast to identify the retrotranslocation factors for both soluble and membrane-bound ERAD substrates (Carvalho et al., 2006). They found that the retrotranslocation of soluble (ERAD-L) substrates usually involved Hrd1p ubiquitin ligase, Hrd3p, Der1 and Usa1p (Hrd1/gp78; Sel1; Der1, 2,3; Herp in mammals respectively) (Fig. 15B). ERAD substrates with misfolded transmembrane segment utilize ERAD-M pathway, while ERAD-C pathway involves Doa10 also (Teb4 in mammals). The ERAD-L pathway is generally the more complex of these three.

An ER membrane complex may be associated with both Yos9p (Os-9) on the lumenal side through the Hdr3 domain and with Cdc48 on the cytosolic side through Ubx2p (Richly et al., 2005; Schuberth and Buchberger, 2005). Some co-immunoprecipitation experiments obtained these data via sucrose gradient centrifugation of digitonin-solubilized membranes. It was proposed that the Hrd1 complex might receive substrates from different sources (Fig.15Bii), since the Hrd3 mutant cells were
still able to degrade CPY* when Hrd1 was over-expressed (Gauss et al., 2006; Plemper et al., 1999a). A newly discovered protein; Usa1 perhaps provides the link between Der-1 and Hrd1 (Figs. 15Bii and iii). Usa1 seemed to associate with all ERAD-L substrates tested in this study and also with Hrd1p (Gauss et al., 2006). Interestingly ERAD-M substrates do not require either Usa1 or Der1 that has been implicated in forming the channel (Carvalho et al., 2006). These results are consistent with other experiments based on yeast mutant strains and the membrane protein version of CPY* (Taxis et al., 2003). ERAD-M substrates might become ubiquitinated by the Doa10 complex that recruits Cdc48 (Neuber et al., 2005; Ravid et al., 2006; Swanson et al., 2001) (Fig. 15Aii).

In summary, the hypothesis is that ERAD substrates undergo both lumenal and cytosolic checkpoints. The first is the cytosolic, and if the protein found misfolded on this side, it becomes ubiquitinated and degraded by the Doa10 machinery. Hence, the cytosolic quality control presumably predominates over the lumenal one. If a membrane protein contains a lumenal or transmembrane misfolded region, the degradation pathway shares similarities with the degradation patterns of soluble ERAD substrates. These observations may result, at least in part, to cytoplasmic chaperones inhibiting the aggregation of the cytoplasmic domains of integral membrane ERAD substrates, while lumenal chaperones may maintain the lumenal ERAD substrates and domains in a non-aggregated state, so that they successfully exit the ER lumen. Everything considered, all of these apparently contradictory findings highlight the need for further experiments (Carvalho et al., 2006) in more well-defined systems.
ERAD of the ubiquitin-proteasome system at the ER membrane

The ubiquitin (Ub) protein exists in all eukaryotic cells in a structurally conserved form as the name entails (Hershko, 1983; Smalle and Vierstra, 2004). Ub has a globular final structure, with five beta-sheet strands forming a cleft into which an α-helix can insert. Free Ubs are conjugated in an ATP-dependent manner to E1 (Ub-activating enzyme) (Hershko, 1983; Hershko et al., 1983) by forming an acyl-phosphoanhydrate bond first between AMP (adenosine monophosphate) and the C-terminal carboxyl group of glycine on the Ub, and later between a thioester bond between E1 and this glycine residue (Hatfield et al., 1997; Kornitzer and Ciechanover, 2000). Transesterification occurs upon transferring Ub from E1 to E2 (Ub-conjugating enzyme). Several isoforms of E2 exist in a cell, but they all have a common domain with a 150-residue region that encloses the active site cysteine. Each E2 has a distinct function in the cell by interacting with a specific E3 (Ub ligating enzyme) (Fang and Weissman, 2004). In the final step, with the help of the E3, the Ub is attached to a lysine ε-amino group on the substrate by an isopeptide bond to the carboxyl group of glycine on the Ub (Fig. 16A). There are at least four different E3 families (Ardley and Robinson, 2005). These include the HECT (homology to E6AP C-terminus) (Huang et al., 1999; Wang and Pickart, 2005), RING/U-box (Real interesting new gene) (Hatakeyama and Nakayama, 2003), APC (anaphase-promoting complex) (Hershko, 2005), and SCF (F-box) protein families (del Pozo and Estelle, 2000; Lechner et al., 2006).
Figure 16. The ubiquitination pathway 26 proteasome and organization and structure. (A) Ubiquitin (Ub) is activated in an adenosine triphosphate (ATP)-dependent manner via the Ub-activating enzyme (E1). Ub is subsequently transferred to an Ub-conjugating enzyme (E2), and ultimately Ub is attached to a lysine in the target protein with by an Ub-ligating enzyme (E3). In some cases another Ub is added to the previous ubiquitin by of the above enzymes in collaboration with E4. Once the polyUb-protein conjugate is ready, it is degraded by the 26S proteasome in an ATP-dependent manner. Individual Ub monomers are released by deubiquitinating enzymes (DUBs) that dismantle the polyUb chain to regenerate both the target protein and Ubs. (b) The possible structure of the 19S regulatory particle (RP) predicted from its individual subunit interactions. The two components, the lid and the base are depicted in pink and light blue, respectively. All six AAA subunits (T) are shown in green, while the other non-ATPase subunits are shown in yellow (N). The lid non-ATPase subunits are in red (N), and all the subunits in the 19S RP are numbered (c) Illustration of the 26S proteasome and its two components, the 20S core protease (CP) and the 19S regulatory particle (RP). (d) The operation of the 20S core protease (CP) postulated from the crystal structure of the yeast 20S CP. The active-site threonines are portrayed in the middle of the CP. The possible functional stages of the 26S are shown throughout the degradation of ubiquitinated proteins. This figure was adapted from (Vierstra, 2003).
The substrate and its conjugate E2 and E3 complexes to determine whether additional Ubs are added to the first Ub conjugated to the substrate to form a polyubiquitinated substrate. Substrates with only one ubiquitin attached are targeted to the lysosome/vacuole. All seven lysines of the Ub can be used to conjugate additional Ub. The most common one is the lysine 48 conjugation, which is recognized by the 26S
proteasome (Kostova and Wolf, 2003; Kruger et al., 2001; Pety de Thozee and Ghislain, 2006).

The first indication that the ubiquitin-proteasome system is involved in the degradation of misfolded ER lumenal and membrane proteins came from studies conducted with a CFTR mutant, CFTR∆F508. Inhibition of the proteasomal 20S subunit resulted in accumulation of CFTR at the ER membrane (Jensen et al., 1995; Ward et al., 1995). Experiments with yeast mutants revealed the presence of five genes [Ufd1p-Ufd5p (Ubiquitin fusion degradation)] that play an important role in ubiquitin-dependent degradation (Johnson et al., 1995). Ufd2p, an E4 multiubiquitination enzyme can further extend the length of the ubiquitin chains (Jentsch and Rumpf, 2007; Rumpf and Jentsch, 2006) (Fig. 16A).

Yeast genetic experiments with temperature-sensitive mutations indicated that Ub-conjugating enzymes Ubc6p and Ubc7p were involved in ERAD (Biederer et al., 1996; Sommer and Jentsch, 1993). Ubc6p is an integral membrane protein, while Ubc7p is a soluble cytoplasmic protein recruited to the ER membrane by an ubiquitin ligase, Cue1p (Fig. 15; cytosolic side) (Biederer et al., 1997).

Substrates degraded via the Ub-proteasome pathway in the ER, include the HMGCoA reductase (3-hydroxy 3-methylglutaryl coenzyme A), which is involved in the mevalonate pathway (Hampton, 2002a; Hampton, 2002b). Hmg2p is one of the ER bound isoforms of this protein, and it is rapidly degraded when a large amount of mevalonate is present.
Hrd1p/Der3p is a complex found in association with the translocon and belongs to the RING-domain Ub-ligase family (Bays et al., 2001a; Deak and Wolf, 2001; Hampton et al., 1996). This complex not only plays a role in Hmg2p degradation, but also in the misfolded carboxypeptidase Y (CPY*) (Bordallo et al., 1998). The Hrd1p/Der3p complex also has at least two Ub-conjugating enzyme partners, Ubc7p and Ubc1p.

A cofactor, Hrd3p has a large lumenal domain that may participate in recruiting ERAD substrates for retrotranslocation (Gardner et al., 2000). Its mammalian homologue, Sel1l, had also been identified and implicated in the degradation of the US11-mediated dislocation of the class I MHC heavy chain. Another Ub-ligase complex in the yeast ER membrane was named Doa1p, and it interacts with both membrane-bound Ubc6p and Cue1p/Ubc7p (Shearer and Hampton, 2004; Shearer and Hampton, 2005).

Since different ERAD substrates require different Ub-ligase/conjugating enzyme complexes, it is very likely that there are several different ERAD pathways present within the ER (Vashist and Ng, 2004). While most of the yeast proteins can also be found in metazoan, there are additional Ub-ligases that participate in mammalian ERAD (Fang et al., 2001; Kikkert et al., 2004; Lenk et al., 2002). For example CHIP, a soluble U-box Ub-ligase has been found to play a role in CFTR degradation (Ballinger et al., 1999; Connell et al., 2001; Cyr et al., 2002; Dai et al., 2005; Jiang et al., 2001; Meacham et al., 2001; Qian et al., 2006; Younger et al., 2006; Younger et al., 2004).

Additional examples are Parkin, a RING-finger containing Ub-ligase, and the Skp1-Cullin1-F-box (SCF) complex (Imai et al., 2002; Imai et al., 2001; Imai et al., 2000).
The latter associates with F-box adaptor proteins (Fbs1, Fbs2) that recognize N-glycosylated proteins that have been dislocated from the ER and targets them for degradation after removal of the N-glycans (Yoshida et al., 2005; Yoshida et al., 2007; Yoshida et al., 2003).

**The 26S proteasome**

Proteasomes are large (2 MDa), multi-catalytic enzyme complexes in the cell that can be found in the cytosol, attached to the ER membrane, or in the nucleus (Peters, 1994; Peters et al., 1994; Smith et al., 2006; Voges et al., 1999) (Fig. 16C). The complete 26S proteasome is composed of 20S core particle that facilitates degradation, and the 19S regulatory particle (RP) cap that recognizes substrates for degradation (Glickman and Maytal, 2002; Glickman et al., 1998a; Glickman et al., 1998b; Glickman et al., 1999; Rubin et al., 1998). Electron microscopy data revealed that the proteasome is around 15 nm in length and 11 nm in diameter (Hase et al., 1980; Hough et al., 1987), cylindrical in shaped, and capped on one or both sides of the 20S core particle by the 19S cap.

The proteasome has a role in several cellular functions, including cell cycle control, metabolic adaptation, and stress response (Finley et al., 1987; King et al., 1996). Proteasomes also have an important role in the cellular immune response. The peptides released from its core chamber after degradation are transported into the ER lumen where they are loaded onto major histocompatibility complexes (MHC)-class I proteins. The resulting MHC-1•peptide complexes are then transported to the cell surface where
they can be recognized by the cytotoxic T lymphocytes (Belich and Trowsdale, 1995; Koopmann et al., 1997; Pamer and Cresswell, 1998; Rivett and Hearn, 2004).

The 20S proteasome is highly conserved among eukaryotes, archaea, and some eubacteria. Prokaryotes have a homologue, called HslV that forms a 240 KDa homododecamer, which becomes a core of the ATP-dependent protease, HslVU (Bochtler et al., 2000; Goldberg et al., 1997; Rohrwild et al., 1997). In archaea, the 20S core particle is comprised of two different gene products (α and β), and two rings of seven β-subunits are sandwiched between two rings of α-subunits (α1-7β1-7β1-7α1-7) (Dahlmann et al., 1986; Hegerl et al., 1991) (Fig. 16D). The α-subunits are not active proteolytically, and they fold as heptamers to serve as a folding template for back-to-back assembly with the seven β-subunits. In yeast, α and β-subunits are all different with their own nomenclature. Four of the seven β-subunits are inactive in yeast, while in archaea all of them are active (Lupas et al., 1995; Wenzel and Baumeister, 1995). The three active β-subunits in yeast recognize different substrate features and have peptidylglutamyl-hydrolyzing (PGPH), trypsin-like, and chymotrypsin like, which explains the broad specificity of the proteasome (Kisselev et al., 2000; Wenzel et al., 1994). Similarly there are at least five different activities in a mammalian proteasome.

The opening of the assembled proteasome does not allow even a 1.4 nm gold particle through, so it is highly restricted, in terms of size. When the protein for degradation eventually enters the main chamber, it is degraded to at least a 4-25 residue long polypeptide, but with the average length of 7-9 residues (Bochtler et al., 1999).
The assembly of the 20S proteasome is a complex process, and passes through several precursor stages over several forms with the help of folding assistants/maturation factors (Hendil et al., 2002; Jager et al., 1999; Mayr et al., 1998; Rodriguez-Vilarino et al., 2000). The 20S proteasome can be activated and inhibited by various molecules. Among the inhibitors are peptide aldehydes (reversible), dipeptidyl boronic acids, peptidyl vinyl sulfanes, and the fungal irreversible inhibitor, lactacystine (Bogyo et al., 1997a; Bogyo et al., 1997b; Fenteany et al., 1995; Kisselev et al., 2006; Klinger and Schubert, 2005; Tsukamoto and Yokosawa, 2006). Polycations, sodium dodecyl sulfate (SDS).

PA28 is an ATP-independent activator of the 20S core particle with about a 200 kDa oligomeric weight (Dick et al., 1996; Groettrup et al., 1996; Ma et al., 1992). PA28 exists in a heterohexameric or heteroheptameric form, composed of two different subunits (α and β). The crystal structure has shown that the central pore of PA28 aligns with the central pore of the 20S, and its C-terminus displaces the N-terminal interdigitating side-chains of the 20S that obstruct its opening (Knowlton et al., 1997). It was also proposed that the PA28 operates in concert with the 19S proteasome cap to feed the substrate into the 20S (Hendil et al., 1998).

The 19S proteasome regulator (PA700 or 19S RP) has a molecular weight of 843 kDa (Etlinger and Goldberg, 1977) (Fig. 16B). One or two 19S can form a higher molecular weight complex with the 20S proteasome in an ATP-dependent manner, to create the 26S proteasome (Rubin et al., 1998). The 19S cap has a ‘V’ shape with dimensions of 15 nm by 20 nm (Peters, 1994; Peters et al., 1993). It is composed of 17
Figure 17. Models depicting the association of PAN with the α ring of the 20S proteasome and how PAN’s C-termini induce gate opening. (A) The C-termini (yellow) of PAN (orange) dock into the intersubunit pores in the top of the 20S. The HbYX motif in PAN’s C-termini is colored red. When PAN associates with the 20S proteasome (upon binding ATP), the translocation channel gate in the 20S is opened. (B) Seven-residue peptides that correspond to PAN’s C-termini dock to the intersubunit pockets in the α ring and induce gate opening by themselves. (C) The multiple ATP-dependent steps in protein degradation and the requirement for nucleotide binding and hydrolysis. This figure is reprinted with permission from Journal of Structural Biology (Smith et al., 2006).
different subunits with the molecular masses of 12-112 kDa (Glickman and Maytal, 2002; Glickman et al., 1998a; Glickman et al., 1998b). The six highly homologous ATPase subunits form a hexameric ring. 19S RP belongs to the AAA (ATPases Associated with a variety of cellular Activities) protein family (Ogura and Tanaka, 2003). The members of this protein family all contain a Walker motif, which is a conserved ATP-binding domain of 200-250 residues (Confalonieri and Duguet, 1995). Most of these AAA protein complexes are involved in ATP-dependent assembling/disassembling, an unfolding/folding protein complexes (Smith et al., 2006). The 19S proteasome can also be divided into two subcomponents called the base and the lid. The base is composed of the six ATPase subunits (Rpt 1-6) and two non-ATPases subunits (Rpn 1-2), while the lid has nine non-ATPase subunits (Rpn 3-12) (Voges et al., 1999). The crystal structure of the 19S complex is not yet known, but the base shares similarity with the Methanococcus ATPase [PAN (proteasome activating nucleotidase)] (Benaroudj and Goldberg, 2000; Zwickl et al., 1999). The PAN complex and the 20S CP together degrade proteins in most archaea in an ATP-dependent manner (Fig. 17A) (Navon and Goldberg, 2001). Elucidation of the structure of PAN by mass spectroscopy and electron microscopy (EM) has revealed some functionally significant insights into the operation of the eukaryotic 19S RP (Smith et al., 2005).

Mutagenetic studies (Glickman et al., 1998b; Glickman et al., 1999) showed that all the six ATPases have distinct and important functions. The Rpt2 (S4) subunit had the most pronounced effect on cell growth and on the opening the gate of the 20S CP (Kohler et al., 2001). It was shown that the association between PAN and the 20S CP
requires ATP, but not ATP hydrolysis (Figs. 17A and C) (Smith et al., 2005). EM studies also revealed that the association between PAN and 20S CP is very similar to the 19S RP and 20S CP without the lid (Benaroudj and Goldberg, 2000). These EM images have shown not only a larger inner ring, but also an outer ring that corresponds to the non-ATPase subunits (Rpn1-2) (Figs. 17A and B) (Kajava, 2002). Perhaps this smaller outer ring of PAN actually corresponds to the coiled-coil domains in both PAN and 19S RP that are thought to play a role in substrate binding and recognition (Gorbea et al., 1999; Zwickl and Baumeister, 1999; Zwickl et al., 1999). PAN binding to the 20S CP was stimulated by the presence of ATP, ATPγS, or AMPPNP, but not by ADP (Smith et al., 2005). Gate opening is the consequence of this binding, and is the key step in activating the 20S CP (Figs. 17A and C). Interestingly, a deletion of the C-terminal HbYX sequence that is present in four of the six 19S RP ATPases, and on all of the PAN subunits, inhibited binding to the 20S CP and subsequent gate opening (Smith et al., 2006). Astonishingly, small peptides containing these seven residues have also been found to facilitate gate opening on their own by inserting themselves into the small pockets between the α-subunits of 20S CP (Fig. 17B) (Smith et al., 2005). It was also established that protein unfolding by 19S RP is distinct mechanistically from translocation into the 20S CP (DeMartino and Slaughter, 1993). These experiments showed that unfolding requires ATP-hydrolysis, but not the translocation (Liu et al., 2006). This finding implied that a protein enters the main chamber passively.

When the directionality of the translocation process was examined, it was found that certain substrates entered the proteasome with the C-terminus, while others entered with
the N-terminus, and some with either end first (Goldberg, 2007). The 20S proteasome can also make peptide cleavages in a polypeptide loop (Liu et al., 2003). The 20S proteasome alone showed no directional bias, so this preference results from an interaction between the substrate and the ATPase subunits (Goldberg, 2007; Navon and Goldberg, 2001). Ubiquitination can also influence the direction by which the substrate enters into the 20S CP (Goldberg et al., 2001).

**AAA protein family: Cdc48/p97**

The AAA protein family belongs to a large and diverse AAA+ protein family that participates in many cellular activities, hence its name: ATPase Associated with various cellular Activities (Erdmann 1991). The AAA+ family includes AAA proteins with protease activity, and includes transcription regulators, activators, and helicases, peroxisomal biogenesis (Pex1, Pex6), vesicle-mediated transport (Sec18/NSF), control of cell division (Cdc48/p97), proteolysis (FtsH), endosome biogenesis (Vps4), and microtubule-separation (katanin) (Aravind et al., 1999; Neuwald, 1999; Neuwald et al., 1999; Volker and Lupas, 2002). AAA proteins contain a well-conserved region, termed second region homology domain (SRH) (Tomoyasu et al., 1993a; Tomoyasu et al., 1993b), and only those proteins that have a homologous region are considered members of the AAA family.

The family has five major subtypes: proteasomal proteins, metallproteases, D1 domains, D2 domains and meiotic proteins (Frohlich, 2001). AAA proteins consist of an N-terminal non-ATPase domain that was proposed to play a role in substrate recognition, and one or two ATPase domains (Fig. 18A). Structures of p97 and NSF (N-
Figure 18. Location of mutated residues lining the pore of p97/VCP. (A) The p97/VCP ADP hexamer is shown in a space-filling view with one of the protomers shown as cartoon (nucleotide as spheres). A side-on view (left panel) is shown with two of the protomer subunits cut away to reveal the central pore. The protomer is colored as follows: N domain, brown; D1 and D2 α/β, blue; D1α, purple; D2α, green. All of the Walker A/B and the SRH residues are near the nucleotide and not near the pore. Loops are highlighted by circles, (I) Arg586/Arg599, (II) Trp551/Phe552, and (III) His317 loop, and are colored red in the space-filling view. The arrow near loop (II) indicates the p97/VCP-Syt1 crosslinked region. The right panel shows a view looking down from the D2 end of the molecule. The location of the Arg586/Arg599 pair is colored red, and a cartoon protomer is given for reference. (B) Detailed views of the pore loops shown both for the protomer (left panels) and the molecular hexamer (right panels). In the hexamer views, protomers are uniquely colored and one of the protomers labeled. This figure is reprinted with permission from Molecular Cell (DeLaBarre et al., 2006) Elsevier (2007).
ethylmaleimide-sensitive factor) have been solved and representing D1 and D2 ATPase
domain proteins, respectively (Fig. 18). When these two structures are superimposed on
each other they display remarkable similarity, although only D1 is active catalytically
(Lenzen et al., 1998; Yu et al., 1998; Zhang et al., 2000).

D1 domain is composed of two regions, one the N-terminal nucleotide-binding site
and the other the C-terminal helical domain. The N-domain consists of three αβα
structures, with a five-stranded parallel β-sheet in the middle. The difference between
the AAA ATPase domain and other P-loop NTPases is the intercalation of the forth β-
strand between strand one and three. As a result, β4 introduces a polar residue between
the Walker B motif and the P-loop. This polar amino acid side chain can interact with
the γ-phosphate group of either ATP or ADP to distinguish the ADP and ATP-bound
states (Hattendorf and Lindquist, 2002a; Hattendorf and Lindquist, 2002b; Karata et al.,
1999). The C-terminal helical domain of this family has more freedom in terms of
structural variety than the N-terminal domain, but it is found in a similar position with
respect to the nucleotide perpendicular to the ATP/ADP base. An arginine residue of the
C-terminus was proposed to interact directly with the nucleotide, but one study reported
that its most important function in Hsp104 is to supply energy rather than differentiate
between ATP and ADP (Hattendorf and Lindquist, 2002b) (Fig. 18B).

AAA proteins are usually assembled into hexameric rings, and each individual
subunit of the ring might contain an ATPase domain. The ATP/ADP binding regions are
arranged in a way that one of the five β-strands (β5) extends towards the ATP/ADP in
the adjacent subunit. Hence, an arginine from each subunit interacts with the preceding
subunit. This arrangement is the so-called arginine finger motif (Ahmadian et al., 1997) that is necessary for complete nucleotide hydrolysis. When ATP is hydrolyzed, an interaction between the γ-phosphate and the polar amino acid is passed on from one ring to another by a movement of the helix between the polar residue within the N-domain and the arginine finger (Schmidt et al., 1999). In some cases, the assembly of the AAA protein is dependent on nucleotide binding and/or interactions with the substrate (Hartman and Vale, 1999). Some AAA proteins have subunits that promote this oligomerization, while a second subunit has only hydrolytic activity (Sec18/NSF and peroxisomal AAA proteins).

The primary question is how these AAA proteins can accomplish their unfolding activity. Presumably the substrate binds to the N-termini of the AAA protein rings. ATP hydrolysis followed by a conformational change in the AAA subunits facilitates the unfolding of the substrate. After this remodeling activity, the AAA protein might release its substrate or transfer it into a central pore of the 20S proteasome or metalloproteases. If the substrate is a membrane protein, AAA proteins appear to first recognize the folding status and length of a soluble, exposed domains of the membrane protein. Then a membrane-spanning segment of the substrate is extracted via the central pore of the AAA protein accompanied by ATP hydrolysis (Chiba et al., 2000; Karata et al., 1999; Kihara et al., 1999; Leonhard et al., 2000; Navon and Goldberg, 2001).

Cdc48/p97 was uncovered over two decades ago while searching for mutants that played a role in cell cycle arrest in yeast. The structure of Cdc48/p97 follows the general theme of AAA proteins. It is composed of a 200 residue-long N-terminal adaptor domain
Figure 19. Schematic picture of the ATP hydrolysis-dependent conformational change in p97. (i) When there is not any nucleotide bound to the complex [D1-red, D2-blue, N-yellow, and the pore is gray (from the top) or dark blue (from the bottom). The color of D2 changes during the graph, which represents structural changes in the D2 ring. The tilted side and bottom of p97 are shown (ii). When ATP binds, the N domain (yellow) moves beneath the level of the D1 ring, the cavity of the D2 ring partly contracts, while the D2 domain turns approximately 20° clockwise. (iii) After ATP is hydrolyzed, the N domain moves back to the plane of the D1 ring as the D2 ring pore opens more (green) with an extra 10° clockwise movement of the D2 ring. (iv) Then the D2 domain turns counter-clockwise about 15° with the narrowing of the D2 hole to the ATP-bound state due to the discharge of the inorganic phosphate from p97 as projections become visible in the middle of the structure (red). This figure was adapted from (Davies et al., 2005).

(N), two 230-250 residue-long ATPase domains (D1 and D2) connected by a short loop, and a C-terminal helical subdomain (C). Six of these proteins are assembled into a
hexameric, double-stacked ring with a small central channel (DeLaBarre and Brunger, 2003; Huyton et al., 2003) that has the N-domain projecting outward.

Cdc48/p97 goes through a large conformational change upon ATP hydrolysis, similar to that of other members of AAA family, and this structural flexibility is responsible for the mechanical force (Fig. 19). During the non-nucleotide bound state, the N-domain protrudes in the plane of the D1 ATPase domain, while the opening of the ring formed by D2 is in its most open state (~70 A°, widest central pore). When ATP binds to both D1 and D2 sites, the N-domain shifts downwards, below the plane of the D1 ring. During this time, the D2 central pore constricts to half its diameter and undergoes a 20° clockwise rotation. After ATP hydrolysis, the N-domain returns to its original position (non-nucleotide bound state), and the D2 central pore opens up again with rotating an additional 10° clockwise rotation. After release of inorganic phosphate, the D2 central pore narrows back again with rotating 15° counterclockwise. When ADP is released, a less compact, but more flexible non-nucleotide bound form is observed (Davies et al., 2005).

There are two important loops lining the central pore that also undergo conformational change during ATP hydrolysis. The first one contains two arginines (Arg586 and Arg599) (Fig. 19B) located at the entrance of the D2 ring that form a double ring facing the central pore in activated ADP-bound state, but not in the ATP-bound and apo state. These may contribute a substrate binding site that is concealed in the ATP-bound state. These two arginines are capable of denaturing any secondary structures and are responsible for protein unfolding activity. These two arginines are also
present in 19S cap of the 26S proteasome, which suggests similar roles for this group in the 19S RP. The second group contains two hydrophobic residues (Phe552 and Trp551) at the interface of D1 and D2 in the center of the pore. A tryptophan mutant of p97 showed decreased affinity for the substrate without any effect on ATP kinetics, suggesting that this residue is a part of a putative hydrophobic patch responsible for substrate binding within the pore. One study proposed (DeLaBarre et al., 2006) that the substrate does not pass through the D1 ring, but only enters D2, and from there it is either removed by the 26S proteasome or exits between D1-D2.

Cdc48/p97 participates in large variety of cellular functions that apparently specified by its abundant cofactors (Richly et al., 2005). These cofactors typically interact with the N-terminal domain (except for Ufd2). Both Cdc48/p97 and some of its cofactors apparently bind simultaneously to the substrate, as shown by their binding to a polyubiquitin chain conjugated to the substrate (Ye, 2005; Ye, 2006; Ye et al., 2001).
CHAPTER II
MATERIALS AND METHODS

Preparation of canine pancreatic microsomes (RM)

Dogs were sacrificed and each pancreas was removed essentially as described earlier (Walter and Blobel, 1983). Pancreas tissue was homogenized and centrifuged for 10 min in an SW34 rotor for 3300 rpm. The fat was removed and the supernatant was centrifuged again for 10 minutes at 10400 rpm in the same rotor. The supernatant was collected and re-centrifuged on a sucrose cushion [50 mM TEA (pH 7.5), 50 mM KOAc, 5 mM Mg(OAc)$_2$, 1.3 M sucrose, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] in a Ti50.2 rotor for 2.5 hr at 40000 rpm. The pellet was resuspended in Buffer AR [50 mM TEA (pH 7.5), 250 mM sucrose, and 1 mM DTT] to a 50 A$_{280}$ units/ml concentration. These microsomes are termed rough microsomes (RMs).

Preparation of column-washed rough microsomes (CRM)

RMs were used to prepare CRM, KRM and SRP as before (Walter and Blobel, 1983). CRMs were prepared by equilibrating Sepharose Cl-2B columns (40 cm length and 5 cm diameter) with Buffer AC [50 mM TEA (pH 7.5), 1.5 mM Mg(OAc)$_2$, 1 mM ethylenediamine tetraacetic acid (EDTA) (pH 7.5), 0.5 mM PMSF, 1 mM reduced glutathione (GSH)] and loading RMs on the top of this column. The microsomes were eluted at a flow rate of 15 ml/hr and 80 drops/tube, collected and centrifuged in a Ti50.2 rotor for 15 min at 23500 rpm at 4 °C. The CRMs were resuspended in Buffer AR with 1 mM GSH. The CRMs were nuclease treated with 20 units/µl Staphylococcus aureus...
nuclease and 1 mM CaCl$_2$ at 23 °C for 10 min. The reaction was stopped by the addition of 2 mM glycol-bis(2-aminoethylether)-N,N',N'-tetraacetic acid (EGTA) (pH 7.5). The CRMs were centrifuged at 34000 rpm for 30 min in a Ti50.2 rotor at 4 °C and resuspended in Buffer AR to a final concentration of 4 equivalent/µl (1 equivalent/µl has an A$_{280}$ of 50).

**Preparation of salt-washed rough microsomes (KRM)**

KRM were obtained after removing the SRP and ribosomes from RM. RMs (8000 equivalents) were thawed and mixed with an equal volume of Buffer AA [50 mM TEA (pH 7.5), 2 mM Mg(OAc)$_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 2% (v/v) aprotinin, and 250 ng/ml protease inhibitor mix]. These RMs were centrifuged in a Type 35 rotor for 30 min at 35000 rpm at 4 °C. The pellets were resuspended in Buffer BB [50 mM TEA (pH 7.5), 1 mM Mg(OAc)$_2$, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1% (v/v) aprotinin, and 250 ng/ml protease inhibitor cocktail] with a Dounce homogenizer. This homogenate was centrifuged again in a Type 35 rotor as before and resuspended in 80 ml of Buffer BB. Then 20 ml of high salt buffer [50 mM TEA (pH 7.5), 20.5 mM Mg(OAc)$_2$, 0.5 mM EDTA (pH 7.5), 1 mM DTT, 2 M KOAc (pH 7.5), 1% (v/v) aprotinin, 100 ng/ml protease inhibitor mix] was added to the 80 ml of washed RMs. The solution was stirred for 15 min and then 17 ml of this was overlayed on a 7 ml sucrose cushion [50 mM TEA (pH 7.5), 5.5 mM Mg(OAc)$_2$, 0.5 mM EDTA, 1 mM DTT, 500 mM KOAc, 350 mM sucrose, 1% (v/v) aprotinin, 100 ng/ml protease inhibitor mix] to centrifuge in a Ti50.2 rotor for 1 hr at 40000 rpm at 4 °C. The pellet was resuspended to a final concentration of 2 equivalent/µl in Buffer AR after Dounce homogenizing it.
twice and flash freezing aliquots of the preparation in liquid N\textsubscript{2} (these membranes are called KRM).

**Preparation of reconstituted microsomes (RRM) and microsomes lacking soluble lumenal proteins (XRM)**

Extraction of soluble lumenal proteins was performed by high pH treatment as described earlier (Bulleid and Freedman, 1988; Haigh and Johnson, 2002; Hamman et al., 1998; Nicchitta and Blobel, 1993). Basically, 200 µl of 1 M concentration of Bicine (pH 9.5-10.0) was added to 200 µl of 2 eq/µl KRM and the volume was adjusted to 1 ml with double distilled water (ddH\textsubscript{2}O). The microsomes were incubated on ice for 30 min, and then layered on 200 µl of sucrose cushion with 50 mM Hepes/50 mM CAPS (pH 9.5) and 0.5 M sucrose. The microsomes were collected by centrifugation with a Beckman TLA 100.2 rotor at 60,000 rpm for 20 min at 4°C. Microsomes were reconstituted by resuspending them in 310 µl Solution A. Solution A was prepared by mixing of the appropriate volume of purified Δgp\textsubscript{αf-BOF} or Δgp\textsubscript{αf} to obtain a final concentration of 5–15 µM with 3.5 mg/ml soluble lumenal proteins or 10-50 µM final concentration selected lumenal proteins in Buffer B [50 mM Hepes (pH 8.0) and 0.25 M sucrose], 2 mM ATP (pH 7.2), and 50 mM of Bicine (pH 10.0) to a final volume of 310 µl, and kept on ice for at least 10 min. The reconstituted microsomes were incubated on ice for 5 min, and the pH was reduced to 7.5 by the addition of 100 µl of 1 M Pipes/Hepes (pH 6.5) using pH paper to ensure the pH was 7.5 +/- 0.1. After 10 min incubation on ice, the microsomes were layered on a 1 ml Buffer A cushion [50 mM Hepes (pH 7.5), 40 mM KOAc, 5 mM MgCl\textsubscript{2} and 0.5 M sucrose] and centrifuged for 20
min at 60000 rpm in a TLA 100.2 rotor. The resulting pellet was thoroughly resuspended in 500 µl Buffer A [50 mM Hepes (pH 7.5), 40 mM KOAc, 5 mM MgCl$_2$] with 0.25 M sucrose. The microsomes were purified by gel filtration chromatography using a Sepharose Cl-2B column (0.7 cm x 50 cm) equilibrated and run in Buffer A using gravity flow and a flow-rate of 0.1 ml/min. Fractions (500 µl) were collected and the microsome-containing fractions were pooled. Fractions containing reconstituted ER microsomes (RRMs) were detected using light scattering ($\lambda_{ex} = 405$, $\lambda_{cx} = 420$).

**Preparation of yeast microsomes for in vitro translation**

Yeast microsomes were prepared by growing yeast [single colony from YPD (20 g peptone, 10 g yeast extract, 20 g dextrose in 1 liter H$_2$O) plate] in YPD media to OD$_{600}$ = 2-6/ml at 30 °C. The cells were harvested by centrifugation at 5000 rpm in a YA-10 rotor for 5 min. Yeast cells were washed in water and resuspended in 10 ml of Buffer YA [100 mM Tris-HCl (pH 9.4), 10 mM DTT]. After an additional spin at 5000 rpm in a JA-10 rotor for 5 min, the pellet was resuspended in 10 ml Buffer YA, incubated at room temperature for 10-20 minutes, centrifuged as above. The pellet was resuspended to an A$_{600}$ of 100 in 0.7 M sorbitol, 10 mM Tris-HCl (pH 7.4), and 0.5% (w/v) glucose in the presence of 10-20 U lyticase/optical density unit of cells. The lysing efficiency was monitored by mixing 1:200 cells in water and measuring the A$_{600}$ until it was less then 5% of the initial value, at which point the cells were placed on an equal volume of sucrose cushion in 1:1 [0.8 M sucrose, 1.5% Ficoll 400, and 50 mM Hepes (pH 7.4)] and centrifuged for 10 min at 6000 rpm in a JA-10 rotor. The spheroplasts were collected after removal of the supernatant and resuspended in lysis buffer [20 mM Hepes/KOH
(pH 6.8), 50 mM KOAc, 100 mM Sorbitol, 2 mM EDTA and 1 mM sorbitol and DTT] to 
A₆₀₀ of 100-150. The spheroplasts were homogenized using a Dounce homogenizer (15 
times up and down). The lysate was centrifuged again in Corex tubes in JA-17 rotor at 
5000 rpm for 10 min. The supernatant was removed and transferred to a polycarbonate 
tube. The supernatant fractions were centrifuged again at 5000 rpm for 10 minutes in JA-
17 rotor at 4 °C and homogenized again using a Dounce homogenizer (15 times up an 
down). The supernatant was spun in JA-17 rotor at 15000 rpm for 10 min at 4 °C and the 
pellet was resuspended in 2 ml Buffer 88 [20 mM Hepes (pH 6.8), 150 mM KOAc, 250 
mM sorbitol, 5 mM MgOAc]. Then the samples was layered on a two step-sucrose 
gradient [20 mM Hepes (pH 6.8), 50 mM KOAc, 1 mM PMSF, 1mM DTT, 2 mM 
EDTA and either 1.5 M or 1.2 M sucrose] centrifuged at 40000 rpm in SW 50.1 rotor at 
4 °C. The microsomes were removed from the interface with a Pasteur pipette. The yeast 
rough microsome (yeast RM) concentration was determined by mixing 5 µ of yeast RMs 
(yeast microsomes) with 2% (w/v) SDS in ddH₂O, and the adjusting volume of the 
sample with Buffer 88 until the final yeast RM A₂₈₀ was 40 (10-12 mg/ml protein 
concentration). The yeast RMs were quick-frozen and stored in aliquots at –80 °C.

**Expression and purification of Δgpαf**

The yeast mating pro-α-factor (Δgpαf) used here lacked a signal sequence, contained 
a C-terminal hexameric histidine tag, and mutations to prevent glycosylation (N23Q, 
N57Q, N67Q), and had a dye attachment site (Y165C or T25C). The Ndel to SalI 
fragment encoding the entire sequence of Δgpαf was cloned into a heat-inducible 
expression vector (pJLA603) (Bush et al., 1991; Schauder et al., 1987). The primers
used for ligation into the vector were GCGCCATATGGCTCAGTCCAACTAC (5’ end) and GCGCGTCGACTTTGTTACATCTACACT (3’ end). Ligation was carried out according to the quick ligation protocol (New England Biolabs, M2200S).

E. Coli BL21 (DE3) cells transformed by this vector were grown at 30°C overnight with shaking to saturation in 100 ml of Luria-Bertani Media (LB) (1 liter LB has 10 g bactotryptone, 10 g yeast Extract, 5 g NaCl) with ampicillin (100 µg/ml). This culture was added the next day to 3 liter LB with ampicillin (100 µg/ml) and grown at 30°C to an A₆₀₀ of 0.6, at which point the expression of Δgpαf was induced at 42°C for 4 hr. Cells were harvested by centrifugation at 4000g for 20 min and then lysed by shaking at room temperature for 1 hr in Buffer AP [8 M urea, 50 mM Hepes (pH 8.0), 100 mM NaCl]. After sedimentation (10000g, 15 min), the supernatant was filtered (Nalgene syringe filter, 0.2 µm; 25 mm) before being loaded on an XK 16/10 Chelating Sepharose Fast Flow column (GE) equilibrated with Co²⁺ and equilibrated in Buffer AP. The column was transferred to a Pharmacia FPLC apparatus and washed with 65 ml of Buffer AP at room temperature. Following another wash with 95% Buffer AP/5% Buffer BP [50 mM Hepes (pH 8.0), 1 M imidazole], Δgpαf was eluted from the column with 300 mM imidazole (55 ml of 70% Buffer AP/30% Buffer BP). A peristaltic pump maintained a flow rate of 3 ml/min during these procedures. The Δgpαf solvent was changed to Buffer CP [50 mM Hepes (pH 8.0), 2 mM EDTA, 1 mM DTT] containing 2 M urea by gel filtration through Sephadex G-25 (30 cm x 2.5 cm i.d.) pre-equilibrated in Buffer CP. This approach for exchanging the solvent was used throughout because the dilution and rapid solvent exchange of this method both reduces the aggregation of
Δgpαf and increases its labeling efficiency when compared to Δgpαf that has been dialyzed.

Δgpαf was further purified by ion exchange chromatography on an FPLC Mono Q-Sepharose column by loading the protein in Buffer CP plus 2 M urea, washing the column with 30 ml of Buffer CP, and then eluting Δgpαf with a 50-ml linear salt gradient (100 - 1000 mM NaCl in Buffer CP). Δgpαf eluted in a single peak near 300 mM NaCl. After Δgpαf was transferred into 20 mM Hepes (pH 8.0), 50 mM NaCl, and 2 mM EDTA by gel filtration to remove DTT prior to labeling, a 4-fold molar excess of 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-S-indacene-3-propionyl)-N'-idoacetylethylene diamine (BODIPY-Fl or BOF, Invitrogen) (Fig. 20) dissolved in ~500 µl DMSO was added dropwise to Δgpαf (10-20 µM; ~30 ml) at 4°C while stirring. The reaction proceeded for at least 12 hr in the dark at 4°C before it was quenched (30 min, 4°C) by the addition of 10 mM DTT. Removing unreacted dyes and transferring the protein into Buffer B [50 mM Hepes (pH 8.0), 250 mM sucrose] was accomplished by first loading a volume of Buffer AP equal to the protein reaction volume on a Sephadex G-25 gel filtration column (25 cm x 2.5 cm i.d.) equilibrated in Buffer B. After the buffer AP had fully entered the resin, the reaction mix was loaded on the column so that the protein was eluted through the urea-containing Buffer AP, thereby releasing any non-covalently bound dye as the protein was finally eluted in Buffer B. The resulting Δgpαf-BOF (5-15 µM) was stored at –80°C in 420 µl aliquots appropriate for reconstitution. No non-covalently bound BODIPY-Fl (BOF) dyes were detected in these preparations by
Figure 20. Structures of BODIPY-Fl IA, BODIPY-Fl SE, and ANB.
fluorescence detection in sodium dodecyl sulfate polyacrylamide gel electrophoresis gel s (SDS-PAGE) using the BioRad FX phosphorimager.

**BOF labeling of BiP, PDI, and GSH**

BiP and PDI were reacted with BOF using the same procedures used for labeling ∆gpαf and were purified by gel filtration from the unreacted dye. GSH (1 mM) was reacted with 10 µM BOF reagent in the same way, but unreacted glutathione was not separated from glutathione-BOF.

**Determining the BOF labeling efficiency**

Analytical HPLC using a C1 column (Poros perfusion chromatography R1/10 4.6 mm diax100mmlength) was employed to separate the unlabeled and BOF-labeled ∆gpαf proteins and to quantify the labeling efficiency. The protein was loaded in Buffer AH [50 mM TEAA [triethylammonium acetate]] and eluted with a gradual increase with Buffer BH (7mM TEAA/ 28 mM CH3CN) (0-80% buffer B in 30 min). The unlabeled protein eluted first followed by the labeled ∆gpαf, and the ratios of the two peaks were ~ 1:1, thus 48-50% of the ∆gpαf was found to be labeled with BOF. Since purifying ∆gpαf-BOF from ∆gpαf proved difficult, the ∆gpαf-BOF used here was only about half-labeled.

The absorbance of the labeled ∆gpαf after removal of the unincorporated dye offered an alternative, although less precise way to determine labeling efficiency. The extinction coefficient of ∆gpαf was calculated by a the number of tryptophan, tyrosine and cysteine residues to be 50640 M⁻¹ cm⁻¹l at 280 nm (Pace et al., 1995), while the published extinction coefficient is of BODIPY-Fl IA is 81000 mol⁻¹ cm⁻¹l in methanol at
503 nm (Invitrogen). The absorbance of ∆gpαf-BOF was measured at 280 nm and 503 nm, and this fraction labeled was determined using extinction coefficients at these wavelengths to obtain the concentrations of ∆gpαf and BOF. The ratio between [∆gpαf] and [BOF] multiplied by a hundred represented the labeling efficiency.

**Purification of BiP and PDI**

Human intein-tagged PDI was purified using a chitin column according to Novagen specifications, while hamster BiP with C-terminal His tags was purified as before (Alder et al., 2005), except using Chelating Sepharose FPLC columns loaded with Co^{2+}. PDI and BiP were each then additionally purified on a Q-Sepharose (GE Healthcare) column using a linear salt gradient. BiP, PDI, and ∆gpαf are each stored in Buffer B; the BiP solution also contained 1 mM ATP. Before use, PDI was incubated at 30°C for 30 min in Buffer A [50 mM Hepes (pH 7.5), 40 mM KOAc, and 5 mM MgCl₂] containing either 5 mM DTT or 5 mM oxidized glutathione (GSSG).

**Preparation of hemoglobin-free reticulocyte lysate**

A 5 ml HisTrap HP (17-5248-01, GE) column was equilibrated with Buffer A at 4°C using a peristaltic pump with a flow rate of 3 ml/min. Reticulocyte lysate (2 ml) was passed through the column at 0.5 ml/min flow rate (generous gift of Dr. William Skach and prepared according to (Carlson et al., 2005). The hemoglobin in the 2 ml lysate normally binds throughout the entire 5 ml column volume without eluting into the flow through. All proteins except hemoglobin were eluted in Buffer A. The hemoglobin was eluted separately in Buffer A plus 1 M imidazole to regenerate the column. The eluted cytosol (8 ml) was filtered (Nalgene syringe filter, 0.2 µm; 25 mm), and 1 mM final
GSH (1M stock) and 1 mM EDTA (pH 7.5) concentration was added. The removal of EDTA is important, because the presence of a chelating agent affects the retrotranslocation of the ERAD substrate. The hemoglobin free cytosol was dialyzed overnight at 4 °C with at least two buffer exchanges against Buffer A with 1 mM GSH. Following dialysis, $A_{280}/A_{260}$ was determined, and either 4 µl of the cytosol without or 1 µl of cytosol with hemoglobin was mixed with 2X SDS PAGE sample buffer to evaluate the globin content. The cytosol without hemoglobin was frozen in 250 µl aliquots and stored at - 80 °C.

**Preparation of wheatgerm**

Nuclease-treated wheat germ was prepared by Yiwei Miao and Yuanlong Shao according to protocols by Erickson and Blobel (Erickson and Blobel, 1983), but the wheat germ was further purified by chromatography using a Sephadex G-25 column and stored in 40 mM HEPES (pH 7.4), 100 mM KOAc (pH 7.5), 5 mM Mg(OAC)$_2$, 2 mM GSH or 2 mM DTT and 100 µM EGTA. The gel filtration step removed any endogenous amino acids that would compete with εANB-Lys-tRNA$^\text{Lys}$ and $[^{35}\text{S}]\text{Met}$ for incorporation into the translation product. Wheat germ extract containing glutathione was used in photocrosslinking experiments to reduce the chemical inactivation of the azide, because the DTT is a stronger reducing agent.
Purification of 26S and 19S proteasome

The 26S and 19S proteasomes used in this study were provided by Dr. George DeMartino and were as described elsewhere (DeMartino, 2005; DeMartino et al., 1994).

Preparation of lumenal proteins

β-Octylglucoside (0.4 M in ddH₂O) was added drop-wise to a final concentration of 20 mM to 4000 equivalents of KRM (Walter and Blobel, 1983) in Buffer C [50 mM Hepes (pH 7.5), 0.25 M sucrose, and 1 mM DTT] with gentle stirring on ice. The detergent-saturated membranes (10 ml) were layered on a 10 ml sucrose cushion [50 mM Hepes (pH 7.5), 0.5 M sucrose] and sedimented (Ti 50.2, 40,000 rpm, 2 hr, 4°C). The supernatant fraction and the upper 2 ml of the sucrose cushion (22 ml) were removed and re-sedimented (Ti 50.2, 40,000 rpm, 5 hr, 4°C). The detergent was removed by extensive dialysis of at least 4 buffer exchanges at 4 °C over 24 hr against 1 liter of Buffer C without DTT each time. The first buffer contained 5 g SM-2 Bio-beads (BioRad) outside the dialysis bag, and was changed after 3 hr. The second buffer exchange also contained Bio-beads outside the dialysis bag, and was dialyzed overnight. The following two exchanges did not contain Bio-beads; only Buffer A without DTT and was allowed to equilibrate for at least 4 hr each. Then the 22 ml of buffer from the dialysis tube containing all lumenal proteins was sedimented again (TLA100.2, 100,000 rpm, 30 min, 4°C). The resulting supernatant (22 ml) containing all of the lumenal proteins was concentrated using Centricon YM-3 filters (Amicon) to 4 ml of ~10 mg/ml being stored at −80°C in 50 µl aliquots and analyzed by SDS-PAGE.
Purification of tRNA and labeling with ANB-NOS and BOF

Purification, aminoacylation and characterization of E.coli tRNA$^{Lys}$ was performed by Y.Shao and Y.Miao as described previously (Flanagan et al., 2003). HPLC-purified Lys-tRNA$^{Lys}$ was chemically modified using then-hydroxysuccinimide ester of N-5-azido-2-nitrobenzoic acid (ANB-NOS; Pierce Chemicals) or BODIPY-Fl (Invitrogen) (Fig. 20) to yield εANB-Lys-tRNA$^{Lys}$ (Krieg et al., 1986) or εBOF-Lys-tRNA$^{Lys}$.

Cell-free translation, translocation, and retrotranslocation

The ppαf gene was cloned by using S. cerevisiae genomic DNA as a template (from Dr. M. Kladde) with PCR primers (5’ end, GCGCGGATCCATGAGATTTCCTTCAATTTTTACTGC; 3’ end, GCGCGAATTCATGAGATTTCCTTCAATTTTTACTGC) into pRSET A vector at the BamHI and EcoRI restriction enzyme sites. pΔgpαf was generated after in vitro mutagenesis of the ppαf DNA (N23Q, N57Q, N67Q).

Full-length mRNA encoding the pΔgpαf was transcribed in vitro using SP6 RNA polymerase with PCR-produced DNA fragments (primers: PAGE-purified 5’ end, GATTTAGGTGACACTATAGAATACCACCATGAGATTTCCTTCAATTTTTACTGC; 3’ end, CTATTAATGATGATGATGATGGTACATTGGTTG (with 6XHis tag)) from the pRSET vector containing full-length ppαf or pΔgpαf.

In vitro translations (350 µl, 26°C, 60 min) contained 140 mM KOAc, 20 mM HEPES (pH 7.5), 2.8 mM MgCl$_2$, 200 µM spermidine, 8 µM S-adenosylmethionine, 1.2 mM ATP, 1.2 mM GTP, 9.6 mM phosphocreatine, 3.5 units of creatine phosphokinase, 30 µM of each of eighteen amino acids (no Met or no Lys), protease inhibitors (0.1 µg/ml final concentration for antpain, pepstatin A, leupeptin, chymostatin; 0.01% (w/v)
for aprotinin; 70 units of RNase inhibitor (Promega), 175 μCi $[^{35}\text{S}]$Met, 70 μl wheat germ cell-free extract, 40 nM canine SRP, 112 equivalents of KRM, and 210 pmoles of εBOF-Lys-tRNA$^{\text{Lys}}$ or Lys-tRNA$^{\text{Lys}}$ (Crowley et al., 1993).

After translation, the microsomal vesicles containing Δgpαf were isolated by centrifugation at 100,000 rpm for 8 min in a TLA 100.2 rotor at 4°C through a 700 μl cushion containing 0.5 M sucrose in Buffer A. The sedimented microsomes were washed and resuspended in 350 μl of Buffer A containing 2 mM ATP, 2 mM GTP, protease inhibitors as above, 16 mM phosphocreatine, 5 units of creatine phosphokinase, and either 60 ml of reticulocyte lysate, 240 ml of reticulocyte lysate without hemoglobin, or no lysate. Samples were then incubated at 30°C and 100 μl aliquots were removed at the indicated times for analysis by SDS-PAGE on a 10-20% gel. Each aliquot was layered on a 100 μl of 0.5 M sucrose cushion in Buffer A, sedimented for 3 min in a TLA 100 rotor at 4°C at 100,000 rpm, and the pellet was resuspended in 40 μl of sample buffer and analyzed by SDS-PAGE on a 10-20% gel. Where indicated, lactacystin [10 mM stock (Sigma) in ddH$_2$O] or epoxomicin [1 mg/ml stock (Calbiochem) in 100% ethanol] was added to the cytosol to a final concentration of 20 μM or 50 μM, respectively, and incubated at 0°C for 30 min before being added to the RRM$s$. Two 5-μl aliquots of each 50 μl sample were removed at various times and precipitated in hot TCA to determine the amount of $[^{35}\text{S}]$Met in polypeptides by collecting the precipitated material on filters and counting the radioactivity in a liquid scintillation counter. A portion (20 μl) of the 50 μl sample was mixed with sample buffer, and analyzed by SDS-
PAGE on a 10-20 % gel. The remaining 20 µl of sample were layered on a 100 µl cushion of Buffer A with 0.5 M sucrose, sedimented for 4 min with TLA 100 rotor at 4°C at 100,000 rpm, and the pellet was resuspended in 25 µl of SDS-PAGE sample buffer. The supernatant and the sucrose cushion were combined, precipitated in hot TCA, and resuspended in 25 µl sample buffer for electrophoresis as above.

The same approach was used with A1PiZ. The A1PiZ cDNA was a generous gift from Dr. J. Brodsky and was cloned into a yeast pYES2 vector. The mRNA was generated from PCR products (primers: PAGE-purified 5’end, GATTTTAGGTGACACTATAGA ATACCACCATGCGTCTT CTGTCTCGTG GGGCATC and 3’ end, TTATTTTTGGG TGGGATTCACCAC) that contained the SP6 polymerase sites.

**Immunoprecipitations**

KRM was isolated by sedimentation prior to immunoprecipitation with affinity-purified antibodies raised against the C-terminal 14 residues of Sec61α (αSec61α; Research Genetics, Huntsville, AL). The membrane pellet was resuspended in 50 µl of resuspension buffer [2% (w/v) SDS, 100mM Tris-HCl (pH 7.5)] by heating at 55°C for 30 min. The samples were completely mixed, added to new 1.5 ml microfuge tubes, and then washed to 500 µl with Buffer AI [140mM NaCl, 10mM Tris-HCl (pH 7.5), 2% (v/v) TritonX-100 (TX-100)] for αSec61α. Individual samples were pre-cleared by adding 40 µl of protein A-Sepharose (Sigma) and rocking at room temperature for 1 hr before the Sepharose beads were removed by sedimentation. The antibodies (5 µg) were added to each sample and rocked at 4°C overnight. On the following day, 40 µl of 2x-
diluted protein A-Sepharose in Buffer AI was added to each sample and the incubation was allowed to continue for another 3 hr at 4°C. The immunoprecipitate was recovered by sedimentation, washed twice with Buffer AI plus detergent, and then washed a final time with the same buffer containing no detergent. Immunoprecipitated material was separated by SDS-PAGE as before (Do et al., 1996) and visualized using a Bio-Rad FX phosphorimager.

For immunoprecipitations using affinity-purified antibodies raised against the C-terminal 13 residues of TRAM (Research Genetics, Huntsville, AL), the C-terminal 50 residues of αDer-1 (Novus Biologicals, Littleton, CO), αPDI, αBiP or αCNX (Calbiochem), the membrane pellet was solubilized in 50 µl of resuspension buffer [1% (w/v) SDS, 100 mM Tris-HCl (pH 7.5)] by heating at 55°C for 30 min. Each sample was diluted to 500 µl with Buffer BI [150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 2% (v/v) Triton X-100, 0.2% (w/v) SDS] and rocked overnight at 4°C after the addition of specific antibodies (5 µg). On the following day, 40µl of 2x-diluted protein A-Sepharose in buffer BI was added to each sample and the incubation was allowed to continue for another 3 hours at 4°C. The immunoprecipitate was recovered by sedimentation, washed twice with Buffer BI, and then washed a final time with the same buffer containing no detergent. The immunoprecipitated material was separated by SDS-PAGE, and visualized using a BioRad FX phosphoimager.
Steady-state fluorescence spectroscopy

Steady-state fluorescence measurements were conducted in an SLM 8100 photon-counting spectrofluorometer using a 450 W xenon lamp light source. The instrument had a double monochromator in the excitation light path, a single emission monochromator, and a Peltier-cooled PMT housing. The quartz cuvettes (Starna Cells, Inc) used in this study were 4 mm x 4 mm. For single wavelength experiments, BOF was excited at 490 nm and the emission was monitored at 513 nm with a band-pass of 4 nm for both excitation and emission. For emission scans, 5 sec integrations of emission intensity were recorded at each specific at 1 nm intervals. The time-dependent emission intensity change was monitored after first thoroughly mixing the sample on ice 20 times using a magnetic stir bar outside the cuvette (Dell et al., 1990). After the cuvette was placed into the temperature equilibrated cuvette-holder in the fluorometer, $F_0$ (zero time-point) was measured immediately. In order to prevent photo-degradation of the fluorescent dyes, the shutters automatically closed and opened before and after data were acquired (every 40 seconds up to typically 2000 second time length).

Static quenching by antibody binding to a fluorophore

This type of quenching occurs when a fluorophores binds to a dye-specific antibody and forms a non-fluorescent complex. When such a dye-antibody complex absorbs light, the dye instantaneously returns to the ground state without the emission of a photon. To ensure that all dyes in the sample were bound to antibodies, the amount of antibody needed to saturate the sample was determined by titration.
Steady-state anisotropy to examine macromolecular association

Anisotropy ($r$) was measured in Buffer A using BOF-labeled protein, Glan-Thompson prism polarizers in L-format as before using a single emission monochromator and a double excitation monochromator (Mutucumarana et al., 1992). The emission intensity was measured at 513 nm by sample excitation at 490 nm through the vertically polarized light and detected via horizontal polarizer as $I_{VH}$. The other parameters ($I_{VV}$, $I_{HV}$, and $I_{VV}$) were determined similarly to $I_{VH}$ after changing the orientation of the polarizers. A blank sample contained the same components as the fluorescent sample, but the BOF dye was omitted from it. The net anisotropy was determined by subtracting the blank intensity (e.g., $I_{VH}$) from sample intensity. The anisotropy was calculated $r = (I_{VV} - G \times I_{VH})/(I_{VV} + 2 \times G \times I_{VH})$, where $G$ is the grating coefficient and equals $I_{HV}/I_{HH}$.

In the anisotropy experiments, 0.5 µM of Δgpαf-BOF was typically incubated in column buffer and gradually titrated with a protein without the fluorescent tag to detect any associations between the fluorescent-labeled and the non-fluorescent-labeled species at 4 °C.

To detect the degradation of Δgpαf-BOF by the 26S proteasome, the anisotropy of the full-length substrate was compared to that of the digested substrate at 30 °C.
**SDS-PAGE**

Translations, photocrosslinking experiments, immunoprecipitation analysis, western blots and purified proteins were analyzed on a 14 cm x 19 cm x 0.8 mm (height x width x thickness) gel via SDS-PAGE. The wells were 2 cm x 0.5 cm x 0.8 mm (depth x width x thickness) and 20-25 µl of samples were loaded into each. The stacking gel was prepared using 4% (w/v) polyacrylamide, 60 mM Tris-HCl (pH 6.8), 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.05% (v/v) N,N,N′,N′-tetramethylethylenediamine (TEMED), and 0.08% (w/v) ammonium persulfate (APS). The resolving gel was prepared by 400 mM Tris-HCl (pH 8.8), 0.08% (w/v) SDS, 0.02% (v/v) TEMED, and 0.08% (w/v) APS and either 15%, 10% or 20% (w/v) acrylamide/bisacrylamide (30:0.8) mixture to obtain a 10-15% or 10-20% gradient gel. The sample was normally mixed with SDS-PAGE running buffer [274 mM Trizma base, 34% (v/v) glycerol, 8.2% (w/v) SDS, 17 mM EDTA] and the samples were heated at 95°C for 5 min unless otherwise specified. The gel was run under constant current (15 mA) for 40 min through the stacking gel and at 32 mA for 2.5 hours for the resolving gel. The wet gel was washed briefly in ddH$_2$O if the gel contained fluorescent-labeled samples that were required to be visualized by BioRad phophoimager immediately after electrophoresis. Otherwise, the gel was placed in a stain solution to detect purified proteins without fluorescent-label or antibodies in the case of immunoprecipitations for at least 10 min [10% (v/v) acetic acid, 50% (v/v) methanol, and 0.125% (w/v) coomassie brilliant blue dye (Sigma)], followed by destaining the gel [10% (v/v) acetic acid, 35% (v/v) methanol] for at least 30 min. The gel was rinsed thoroughly in water and dried on a
3MM Whatman paper for 40 min on a BioRad gel-drier. The gel was then exposed to a Kodak phophorimaging screen overnight or longer, and visualized using the BioRad FX Molecular Imager and its Quantity One software.

**Western transfer and immunoblotting**

Following SDS-PAGE, gels transferred to PDVF membrane (Amersham, catalog # RPN303F) using the tank transfer method. Blots were treated with blocking solution [3% non fat dried milk, 50mM Tris-HCl (7.4), 150mM NaCl, 0.05% (v/v) Tween-20] for 30 min at room temperature, then incubated with primary antibody (diluted in blocking solution) for either 2 hr at room temperature, or overnight at 4°C. Then the blots were washed three times (10 min per wash, room temperature) with TBS-T [50mM Tris-HCl (pH 7.4), 150mM NaCl, 0.05% Tween-20], followed by incubation with the secondary antibody conjugated to horseradish peroxidase (HRP) (1:10000, blocking solution, 1 hr, room temperature). The blots were washed three times with TBS-T, and developed using the ECL+Plus chemiluminescence reagent kit (GE healthcare, RPN2132) according to instructions and the blot was detected using the BioRad phosphoimager.

**Hot TCA assays**

Radioactive proteins were translated in vitro (next section) whenever conditions for translation and translocation were optimized, and also when degradation of a protein over time was assessed. To quantify the amount of acid-precipitable radioactivity in a sample, aliquots were incubated in hot acid (protein precipitated in hot acid, while DNA and RNA are chemically degraded). In general, 2 µl of a translation/translocation reaction was removed and mixed with 1 ml of 10% (w/v) trichloroacetic acid (TCA), and
3% (w/v) casamino acids (CAA) in a 13 x 100 mm glass tube. The samples were heated at 85 °C for 10 min and then cooled on ice for 2 min. The heating hydrolyzed all the RNA molecules, but precipitated the proteins. The samples were vortexed and then filtered under vacuum through a 25 mm Metricel membrane filter (0.45 µm pore size, Gelman Sciences), which has been wetted previously with 3 ml of ice cold 5% (w/v) TCA. The samples on the filter were also washed with 5% TCA three times and then dried under an infrared heat lamp for 10 min, resuspended in PPO/POPOP/toluene scintillation cocktail and counted in a Beckman scintillation counting apparatus.

TCA precipitation was also used to recover proteins prior to SDS-PAGE. When a translation was completed, an equal volume of 25% (w/v) cold TCA was added to the tube, left on ice for 30 min, and sedimented at 15000 rpm for 10 min at 4 °C in a microcentrifuge tube. The supernatant was removed by aspiration, and the pellet was washed with 1 ml of acidic acetone (19:1 acetone: 0.1 M HCl), followed by recentrifugation for 5 min at 15000 rpm. The pellet was dried by vacuum centrifugation on the Speed-Vac and mixed with SDS-PAGE sample buffer.
CHAPTER III

RETROTRANSLOCATION OF AN IN VITRO TRANSLATED ERAD SUBSTRATE FROM CANINE PANCREATIC AND YEAST MICROSONES

Background and experimental design

The ultimate goal of this project was to create a retrotranslocation assay that was based on the well-established protocol for reconstituting canine pancreatic microsomes with selected proteins or small molecules (Alder et al., 2005; Bulleid and Freedman, 1988; Haigh and Johnson, 2002; Hamman et al., 1998; Nicchitta and Blobel, 1993).

The yeast mating pheromone prepro-α-factor (ppαf) is composed of a 19 amino-acid long N-terminal cleavable signal sequence, a 64 amino-acid long pro-region that contains three consensus N-glycosylation sites and a carboxyl-terminal domain with four 13 amino-acid repeats (see later). This protein obtains modified N-glycans in the yeast Golgi apparatus, characteristic to yeast glycoproteins, when high mannose chains are added to its three core glycans. Later in this pathway, the basic spacer residues between the four repeat segments serve as cleavage sites for specific proteases (KEX2, STE13, KEX1), which separate them to form four fully matured α-factors for secretion from the cell. The proregion of ppαf might direct this protein to the proper compartment within the secretory pathway and facilitates the proteolytic processing by KEX2 of this protein (Kurjan and Herskowitz, 1982). As all other secretory proteins are, ppαf is targeted to the ER membrane post-translationally in yeast and co-translationally in mammalian cells (Garcia and Walter, 1988; Hansen et al., 1986). This finding suggests that this yeast protein translocates across the ER membrane by a different mechanism in mammalian
and yeast cells, which may also be the case during its retrotranslocation. These ppαf targeting and translocation differences between yeast and mammalian systems might be attributable to the SRP dependence of ppαf trafficking in mammalian cells. Other yeast secretory proteins used in this study did not translocate through the mammalian membrane post-translationally, even in the presence of SRP (Hansen et al., 1986), an observation that contrasted with ppαf translocation. Another interesting observation made in this paper was that the fully glycosylated version of ppf (3gpαf or gpαf) translocated into canine RMs had a slower mobility on an SDS-PAGE gel did those translocated into yeast RMs.

Experiments conducted in a rat pituitary cell line that also expressed a mammalian homologue of the KEX2 enzyme presented some very interesting findings regarding the secretion of this protein in a mammalian system (Su et al., 1993). The wild-type protein (pgpαf) was targeted properly to the mammalian ER membrane in vivo, but never reached the Golgi apparatus. Further examination of the differently glycosylated ppαf as using various inhibitors revealed the fate of this protein in mammalian systems compared to yeast. In this study, the authors concluded that both gpαf and Δgpαf degraded in vivo in the early secretory pathway in mammalian cells, proposing the interesting possibility that this yeast protein was recognized as a non-native substrate in a heterologous system even of the wild-type configuration, and eliminated. This group revealed that ppαf interestingly did not interact with the mammalian BiP or Grp94 because they did not co-immunoprecipitate together. These data were contrary to several findings in yeast (Kabani et al., 2003) that demonstrated the dependence of ppαf on
Kar2p (yeastHsp70) either for translocation into the yeast ER or for degradation of its mutant form (Matlack et al., 1999).

In yeast, both in vivo and in vitro experiments demonstrated that if glycosylation of pαf is prevented, pαf becomes an ERAD substrate (Δgpαf) (Brodsky et al., 1993; Nishikawa et al., 2001; Pilon et al., 1998) and is retrotranslocated to the cytosol for degradation. However, currently there are no data that would suggest that a mammalian in vitro system would support the retrotranslocation of Δgpαf, one of the most studied ERAD substrates. It was suggested that a portion of Δgpαf (mΔgpαf) could become O-glycosylated in the ER lumen in yeast, which might inhibit its retrotranslocation from the lumen (Harty et al., 2001; Nakatsukasa et al., 2004).

A number of other potential ERAD substrates are currently available. The mutant Z variant of α-1-proteinase inhibitor (A1PiZ, also known as α-1-antitrypsin-Z or AT-Z) is one of the soluble substrates for ERAD that degraded both in ubiquitination dependent and independent manner. The mutated form of yeast carboxypeptidase Y (CPY*) is another favored ERAD substrate. CPY* uses an ubiquitination-dependent degradation pathway and might use different degradation routes to exit from the mammalian ER (Mancini et al., 2003). A number of toxins have been shown to exploit the retrotranslocation machinery of their target cells to gain entry into the cytosol, but avoid subsequent degradation, e.g. protein ricin, a member of the AB-toxin family (Hazes and Read, 1997; Simpson et al., 1999).
Retrotranslocation of $^{35}$S-labeled Δgpαf from KRMs in the presence of cytosol

Before a more complicated reconstitution experiments with canine microsomes were examined, it was necessary to determine whether Δgpαf retrotranslocated in vitro from intact KRMs and also whether the experiments with yeast RMs were reproducible (Gillece et al., 1999; McCracken and Brodsky, 1996). The previous procedure for analyzing retrotranslocation of radiolabeled Δgpαf lacking the three consensus glycosylation acceptor sites (N23Q, N57Q, and N67Q) was therefore modified, using both canine KRMs and yeast RMs with mammalian cytosol in our system.

First, KRMs were used in a wheat germ in vitro translation system that synthesized full-length Δgpαf. This protein was translocated into these microsomes in the presence of mammalian SRP. The vesicles were sedimented at the end of the translation, washed with standard buffer (SB) [50 mM Hepes (pH 7.5), 40 mM KOAc, 5 mM MgCl$_2$] and resuspended in SB either with cytosol or without [in the presence or absence of proteasome inhibitors] to initiate retrotranslocation. The samples were mixed and kept on ice until retrotranslocation was initiated at 30 °C. An energy-generating system (EGS), which contains ATP, creatine phosphokinase and phosphocreatine and protease inhibitors (which do not have any effect on the proteasomes) were also added to the ERAD assay, unless the dependence on ATP was investigated. These experiments determined how much Δgpαf has remained in the microsomes, after various times of retrotranslocation to the cytosol (Fig. 21). When Δgpαf is translocated into the ER lumen, its signal sequence is cleaved off. This yields a higher mobility radioactive species on the SDS-PAGE gels (Fig. 21; lower band). Signal sequence cleavage also
Figure 21. Retrotranslocation of $^{35}$S-labeled Δgpαf in a mammalian cell-free system. Cytosol- and ATP-dependent degradation of Δgpαf was accomplished by the retrotranslocation and processing of pΔgpαf. Translation reactions containing KRM and $[^{35}$S]Met synthesized radiolabeled Δgpαf, and degradation of Δgpαf was then monitored by 0’, 10’, 30’, and 60’ min chase incubation of the resulting KRM after sedimentation and resuspension in the presence or absence of cytosol, ATP (lanes 1-12 and 21-24) or apyrase (13-20), or proteasome inhibitors (21-24). All lanes show the microsomal fractions from the total reactions. The radioactive samples were run on a SDS-PAGE gel and visualized by BioRad FX phosphorimager.

indicates the fraction of proteins that is translocated into the microsomes. Signal sequence cleavage does not occur if hydrophobic pΔgpαf associates with the cytosolic side of the ER membrane in vitro and is not translocated, as often the case. Also, some
full length pΔgpαf that may have been translocated but not signal cleaved because of inefficient signal peptidase activity (Fig. 21; upper band).

After microsomes were resuspended and incubated in the presence or absence of cytosol, aliquots were removed at various time-points. These aliquots were then used to assess the amount of Δgpαf present still in the KRM or alternatively retrotranslocated into the cytosol, where Δgpαf was either degraded or stabilized if the proteasome function was impaired. Δgpαf was retrotranslocated in the presence mammalian cytosol (hemin-free rabbit reticulocyte lysate) (Fig. 21; lanes 1-4) and EGS, because the amount of full length Δgpαf decreased over time. The presence of a small amount of DMSO (the solvent for the proteasome inhibitor, MG132) did not affect ERAD, but it was a necessary control to show the effect of MG132 was not due to the solvent (Fig. 21; lane 5-8). In the absence of cytosol, retrotranslocation did not occur and Δgpαf was not extracted from the microsomes (Fig. 21; lanes 13-16). When apyrase was present in two different concentrations (15 U/ml or 30 U/ml), very little retrotranslocation was observed. The small quantity of retrotranslocated and degraded Δgpαf might be due to some ATP still present in the microsomes or bound to some cytosolic ATPases. These data suggest that Δgpαf is retrotranslocated from mammalian microsomes in a cytosol- and ATP-dependent manner.
Retrotranslocation of S$^{35}$-labeled Δgpαf from yeast RMs in the presence of mammalian cytosol

Yeast RMs were also tested for ERAD activity (Fig. 22), but with mammalian cytosol instead of yeast cytosol (McCracken and Brodsky, 1996). The data obtained showed Δgpαf was retrotranslocated from yeast RMs in the presence of mammalian cytosol (Fig. 22; lanes 1-3), but that this ERAD substrate remained in the microsomes in the absence of cytosol (Fig. 22; lanes 4-6).

Figure 22. Retrotranslocation of S$^{35}$-labeled Δgpαf from yeast RMs in the presence of mammalian cytosol and EGS. Cytosol-dependent retrotranslocation of Δgpαf from yeast RMs in the presence of mammalian cytosol was accomplished by translocating the full length protein in wheat germ extract without the addition of any SRP, but in the presence of yeast RMs. After translocation, these vesicles were washed SB and resuspended in +/- cytosol. Aliquots were removed at the designated time points and TCA precipitated to assess the retrotranslocation and degradation of Δgpαf. A cytosol dependent retrotranslocation was observed (lane 1-3) time period concluded from the disappearance of the lower band over 20’. Some non-translocated or pΔgpαf that was translocated but not cleaved by signal peptidase was also present in these reactions (upper band). In the absence of cytosol, retrotranslocation did not occur (lanes 4-6). The radioactive samples were run on a SDS-PAGE gel and visualized by BioRad FX phosphorimager.
Figure 23. Retrotranslocation and degradation of Δgpαf-BOF in the presence of lactacystine. 

pΔgpαf was translated in vitro in wheat germ with εBOF-Lys-tRNA\(^{\text{Lys}}\) and \[^{35}\text{S}\] Met, and translocated into KRMs. The washed vesicles were resuspended into cytosol (panels A and B; lanes 4-6), cytosol treated with lactacystine (panels A and B; lanes 7-9), or cytosol containing Hb in (panels A and B; lanes 1-3), or SB (lanes panels A and B; lanes 10-12). At the designated time-points, aliquots were removed and TCA precipitated to assess degradation by detection with radioactivity (A), or fluorescence (B). Only the cytosolic fraction is shown in C, which was prepared by removing a fraction from the total samples, sedimenting the microsomes and precipitating the supernatant in TCA. The radioactive and fluorescence samples were run on a SDS-PAGE gel and visualized by BioRad FX phophorimager.
Retrotranslocation of a fluorescent-labeled ERAD substrate in the presence of proteasome inhibitors

A similar approach was used as discussed previously in this chapter (Fig. 21) to evaluate the effect of lactacystine (an irreversible proteasome inhibitor) on degradation of the retrotranslocated Δgpαf (Fig. 23). Samples were TCA-precipitated after removing aliquots at different times and to evaluate the extent of degradation. These Δgpαf had also a BOF dye incorporated because εBOF-Lys-tRNA_Lys was added to the translation mixture. These experiments demonstrated that the presence of a BOF dye did not detectably affect or alter the rate of Δgpαf retrotranslocation (Figs. 23 and 24). The Δgpαf degradation was almost fully inhibited (Fig. 23; lanes 7-9) when the cytosol was treated with lactacystine (an irreversible proteasome inhibitor), as detected by both radioactivity (Fig. 23A) and fluorescence (Fig. 23B). Δgpαf also remained stable in the absence of cytosol, as it was observed with only in the microsomal fractions (Fig. 21; lanes 10-12). When the samples were sedimented at each time point to separate the cytosolic and microsomal fractions, the presence of retrotranslocated, and nondegraded
Figure 24. Retrotranslocation and degradation of Δgpαf and Δgpαf-BOF in cytosol with or without hemoglobin. The mRNA for pΔgpαf was translated in the presence of wheat germ extract, [35S] Met, SRP and either εBOF-Lys-tRNA^{Lys} (lanes 1-9) or only Lys-tRNA^{Lys} (lanes 10-12). Reticulocyte lysate (cytosol, lanes 1-3), lysate that lacked hemoglobin (cytosol-Hb, lanes 4-6 and 10-12), or no cytosol (lanes 7-9) were compared. The average percentages of Δgpαf-BOF and Δgpαf are shown below and normalized to zero time point after quantification by detection either radioactivity (A) or BOF fluorescence (n=7 and standard errors are 2-7%).
Δgpαf was apparent in the cytosolic supernatant fraction (Fig. 23C; lanes 4-6). The lower band in Fig. 23C is the signal cleaved Δgpαf, while the top band is the non-translocated pΔgpαf that remained in the cytosol. These results revealed that Δgpαf was retrotranslocated back into the cytosol for degradation, but that this degradation step can be prevented if the 20S subunit of the proteasome is inhibited. This data are consistent with previous experiments (Fig. 21; lanes 16-18), although a less potent and reversible inhibitor was used. The effect of BOF was also investigated (Fig. 24). When Δgpαf was translocated into KRMs with or without a BOF dye attached (Fig. 24A; lanes 4-6 and 10-12) the retrotranslocation and degradation rates were very similar for Δgpαf and Δgpαf-BOF. This implies that the presence of fluorescent BOF dye did not alter ERAD rate.

**Retrotranslocation of wild type 3gpαf from KRMs, but not from yeast RMs**

One interesting aspect of pαf secretion in a mammalian system that this protein is recognized as an ERAD substrate not only in the mutant, non-glycosylated structure (Δgpαf), but also in its glycosylated, wild type form (3gpαf). Experiments were conducted as described earlier (Fig. 21) with the in vitro translated, wild type ppoαf mRNA. When KRMs that contained 3gpαf were incubated with cytosol and EGS, a time-dependent retrotranslocation was observed (Fig. 25; lanes 1-6). On the contrary, yeast RMs recognized 3gpαf as a properly folded and processed protein, not an ERAD substrate, and it was therefore not retrotranslocated (Fig. 25; lanes 7-9). Most of the translocated pαf had all three glycans on (3gpαf), but some possessed either two (2gpαf)
Figure 25. Retrotranslocation and degradation of gpαf. The ppαf mRNA was translated and translocated into KRMs (lanes 1-6) or yeast RMs (lanes 7-9). These vesicles were sedimented and resuspended in EGS and mammalian cytosol (Ret, reticulocyte lysate) (lanes 1-3 and 7-9) or in the absence of cytosol (lanes 4-6). After incubation at 30 °C, aliquots were removed at the indicated times, sedimented, and pellets were resuspended in SDS-PAGE running buffer. The gel was visualized by radioactivity with BioRad phophorimager.

or one glycans (1gpαf). The non-glycosylated pαf is also detectable on this gel (Fig. 25; lanes 1-6, black triangle) and showed a cytosol-dependent degradation over-time, which consistent with the data depicted earlier (Fig. 21).

Yeast RMs and KRMs have different glycan processing machineries

In order to further investigate why the wild 3gpαf is recognized in the mammalian system as an ERAD substrate, the glycosylation patterns of the 3gpαf in both yeast RMs and KRMs were examined. The translocation products were run on an SDS-PAGE gel beside each other, using two different batches of yeast and KRM to show that the results were not batch specific (Fig. 26; Lanes 1-4)
Figure 26. Differential trimming of 3gpαf in yeast and mammalian microsomes. The ppαf mRNA was translated and translocated into yeast RMs (lanes 1-2) or KRM (lanes 3-4). After translocation, the membranes were resuspended in SDS-PAGE running buffer and run on a 10-20% SDS-PAGE gel. The gel was visualized by radioactivity with BioRad phosphorimager. Experiments in lanes 1 and 2 were done with two different batches of microsomes (in lane 1 the yeast RM was purified in this lab as described in the methods section; in lane 2 the microsomes were generously provided by Dr. Brodsky), but the two batches show the same glycan processing. Mammalian microsomes in lanes 3-4 were used from different microsomal preparations, made in this lab.

The SDS-PAGE results revealed that 3gpαf had different mobilities in KRM versus yeast RMs (Fig. 26; lanes 1-2 vs. 3-4). This discovery implied that the three glycans on 3gpαf were processed or trimmed in the mammalian ER differently compared to the yeast ER. This glycan processing difference between these two systems is probably the result of an extra glucose residue that is added back to or not removed from branch A on
any of the three carbohydrate moieties via UGGT (Fig. 8). UGGT preferentially reglucosylates hydrophobic, unfolded proteins in the CNX/Calreticulin (CRT) cycle and is absent in yeast RMs.

**Retrotranslocation of S$^{35}$- labeled Δgpαf from reconstituted RMs (RRMs) in the presence of cytosol and EGS**

After the investigation of retrotranslocation of Δgpαf from intact microsomes (KRM), the retrotranslocation competence of RRM was investigated. RRM has a fully functional forward translocation activity and they are resealed in the correct orientation after lowering the pH back to 7.5. Ultimately, a purified ERAD substrate was going to be encapsulated into these microsomes, so it was crucial to show that RRM were able to carry out retrotranslocation.

RRM reconstituted with total with lumenal content (Fig. 27; lanes 1-6) or without any soluble proteins (XRM) (Fig. 27; lanes 7-9) were used for in vitro translocation of the wheat germ translated pΔgpαf. This ERAD substrate was retrotranslocated from RRM (Fig. 27; lanes 1-3, bottom band) in a cytosol dependent manner (Fig. 27; lanes 4-6, bottom band). The XRM that did not contain soluble luminal proteins did not support retrotranslocation as had the RRM with a full set of soluble luminal proteins present (Fig. 27; lanes 7-9 bottom band). On the other hand, pΔgpαf was translocated into RRM and XRM to similar extents.
Figure 27. Retrotranslocation of $p\Delta gp\alpha f$ from reconstituted microsomes. The $p\Delta gp\alpha f$ mRNA was translated and $\Delta gp\alpha f$ translocated into RRM (lanes 1-6) or XRM (lanes 7-9). These vesicles were sedimented and resuspended in mammalian cytosol (Ret, reticulocyte lysate) (lanes 1-3 and 7-9) or in the absence of cytosol (lanes 4-6), EGS and incubated at 30 °C. Aliquots were removed at the indicated time-points, sedimented, and the pellet was resuspended in SDS-PAGE running buffer. The gel was visualized by radioactivity with BioRad phosphorimager.

Can another ERAD substrate retrotranslocate from KRM?

An alternative ERAD substrate is the mammalian misfolded $\alpha 1$-antitrypsin inhibitor, which has several misfolded variants, but in this study the Z-variant (A1PiZ) was examined. A1PiZ has a mutation at position 342 where a negatively charged glutamic acid has been replaced by a positively charged lysine residue. This mutation causes misfolding, retention in the ER and eventually degradation in the cytosol. A1PiZ has three glycosylation sites and no disulfide bonds. When pA1PiZ mRNA was translated and translocated into KRM, the largest fraction of A1PiZ became glycosylated completely (three carbohydrate moieties), but there were also double, single and
Figure 28. Retrotranslocation of A1PiZ from KRM. A1PiZ was translated in the presence of wheat germ extract and [35S] Met and KRM. The sedimented microsomes were resuspended with (lanes 4-6 and 10-15) or without (lanes 1-3 and 7-9) cytosol (Ret) and with EGS (lanes 1-6, 7-9, and 13-15) or apyrase (lanes 10-12). Fractions were removed at the indicated times and either further purified by sedimentation to remove the cytosolic part (Lanes 1-6). The complete samples, including cytosolic proteins were TCA precipitated to assess degradation (Lanes 7-15). A non-glycosylated pA1PiZ and A1PiZ were run in lane 16 as controls. The samples were resuspended in SDS-PAGE running buffer. The gel was visualized by radioactivity with BioRad phosphorimager.
nonglycosylated species present (Fig. 28; lanes 1-16). These KRMs with A1PiZ inside (1-3gA1PiZ and the signal cleaved A1PiZ) were washed and resuspended in cytosol and EGS to assess whether retrotranslocation of this protein occurs in vitro. When both the pellet and the total (pellet and supernatant together) fractions were examined individually for degradation during 60 minutes time-course, it was found that the amount of A1PiZ and especially the single glycosylated form (1gA1PiZ) decreased. The triple, double and nonglycosylated species also appeared to be degraded (Fig. 28; lanes 13-15). This degradation was cytosol and ATP-dependent, because in the absence of these two components no proteolytic activity was observed (Fig. 28; lanes 7-12). When the microsomal (pellet) fractions were examined alone after separating the cytosol from the pellet by sucrose gradient centrifugation, a cytosol-dependent decrease was observed in the amount of A1PiZ present in the microsomes. This finding showed that 0-3gA1PiZ was retrotranslocated from the KRMs only in the presence of cytosol (Fig. 28; lanes 1-6), perhaps in an ubiquitination-independent manner.

**Retrotranslocation of an in vitro translated ERAD substrate from KRMs detected by fluorescence spectroscopy**

The retrotranslocation of ∆gpαf was also examined in the fluorometer. These experiments were necessary to establish the viability of the approach before doing more complex experiments with the purified ∆gpαf-BOF. The ∆gpαf mRNA was translated in the presence of KRMs, SRP, and either εBOF-Lys-tRNA_{Lys}, or Lys-tRNA_{Lys} as a dye free background sample. KRMs were then purified on a gel-filtration column to separate the microsomes from the non-translocated pΔgpαf-BOF and from εBOF-Lys-tRNA_{Lys}. 
Figure 29. Retrotranslocation of the in vitro translated Δgpαf-BOF from KRM5 detected by 
antibody quenching. The mRNA for pΔgpαf was translated in the presence of wheat germ extract, [35S] 
Met, SRP and either εBOF-Lys-tRNA Lys for the fluorescent sample or only Lys-tRNA Lys for the blank 
samples. The KRM5 containing the in vitro translated Δgpαf-BOF were purified by gel-filtration and 
resuspended in cytosol-Hb. The net emission intensities of samples with and without αBOF were recorded 
as a function of time and then normalized (F = F +αBOF / F −αBOF). F0 is the initial net normalized intensity at 
t0, while F is the net normalized intensity at any time t. The average F at each time point for three separate 
experiments is plotted; error bars show the standard error for each point and dictate line thickness.

The microsomes were collected and placed into a fluorescence cuvette in the absence or 
presence of cytosol, BOF-specific antibodies (αBOF) and EGS for fluorescence 
experiments. The emission intensity was recorded in the presence and absence of αBOF 
for 500 seconds (Fig. 29). The magnitude of the αBOF-dependent intensity change was 
then given by the ratio of intensities with and without αBOF (F = F +αBOF / F −αBOF * 100 
or % F/F0). Only the αBOF-dependent intensity is shown here. A cytosol dependent
decrease in fluorescence intensity was observed over time (Fig. 29; circles) without subtraction of the non-retrotranslocation dependent decrease in fluorescence intensity (explained later in Chapter IV). These data suggested that some Δgpαf-BOF was transported from the ER lumen into the cytosol where the dye was bound by αBOF and quenched. The reduction in intensity totaled 10% by the end of the 500-second period; while no significant quenching was observed in the absence of cytosol (Fig. 29; triangles).

**Discussion**

Several important conclusions can be drawn from these experiments. First, the mammalian system recognizes the misfolded Δgpαf as an ERAD substrate and retrotranslocates it back to the cytosol. Second, a BOF dye covalently conjugated to Δgpαf does not alter its retrotranslocation rate (Figs. 23 and 24). Third, Δgpαf, 3gpαf and A1PiZ were degraded in the cytosol by the proteasome, because their degradation was not affected by protease inhibitors, only by proteasome inhibitors. Fourth, retrotranslocation also was an ATP-dependent process, because the addition of apyrase that eliminates any ATP present by hydrolyzing ATP into AMP and pyrophosphate (Figs. 21, 23, 24, 28) prevented ERAD to occur in at least two substrates.

The wild-type 3gpαf was also recognized by the quality control system in the mammalian ER, but not by the yeast ER (Fig. 25). One can easily detect differences in the mobilities on the SDS-PAGE gel between 3gpαf present either in KRM or yeast RMs. Moreover, the glycosylation patterns of 3gpαf in either a mammalian or a yeast ER showed some differences (Fig. 26). The same observation was made earlier by a
group that was investigating the post-translational translocation of ppαf into yeast RMs versus canine RMs (Hansen et al., 1986). These distinctions in glycan processing between yeast and canine ER may result from a lag in the glycan processing within the mammalian ER. During this time period, the newly translated/translocated protein goes through a refolding attempt by the CNX/CRT cycle in which glucose is added back to the A-chain by UGGT (Fig. 8). The presence of extra glucoses on all three of the N-linked carbohydrate moieties of 3gpαf may account for the different mobility in the SDS-PAGE gel. Since Saccharomyces cerevisiae does not contain UGGT or its homologue, it is quite likely that 3gpαf avoids this type of a quality control in yeast. In contrast, the unglycosylated Δgpαf appears to take similar routes in both yeast and mammals, because both systems recognized Δgpαf as an ERAD substrate.

The degradation of 3gpαf was also noted in an in vivo study (Su et al., 1993) that tried to use a mammalian system [rat pituitary cell line] to investigate the biogenesis of ppαf. A mammalian system seemed ideal to examine the movement of 3gpαf from the ER to the plasma membrane, since trafficking through the mammalian secretory pathways takes much longer than in yeast. These in vivo experiments also showed that 3gpαf becomes stabilized in mammalian cell lines that contained mannosidase inhibitors, while these inhibitors had naturally no affect on the retrotranslocation of Δgpαf (Su et al., 1993). This observation is consistent with the hypothesis that proposes that mannose trimming is very important in the quality control of glycoproteins in a mammalian system (Yoshida, 2003). This so-called “timer” mechanism would allow
improperly folded glycoproteins to go through extensive refolding cycles to reach the correct conformation. If these attempts fail, a mannose residue is removed from the B-chain of the carbohydrate moiety (Fig. 8), which may facilitate the interactions with mannosidase-like chaperones (Fig. 14) (Hirao et al., 2006). If folding is unsuccessful, eventually these glycoproteins are targeted for degradation (Helenius and Aebi, 2004).

Interestingly, some of the ∆gpαf was found to become O-mannosylated in yeast RMs in vitro, supposedly to escape retrotranslocation (Harty et al., 2001). O-mannosylation starts with the addition of dolichol-phosphate mannose to a serine or threonine residue on the protein by one of the seven-mannosidase transferase (Pmt1-7p). This research group also found a set of O-mannosylated forms (m∆gpαf) crosslinked to the Sec61 channel, which led them to conclude that the translocon also acts as a retrotranslocon. It was suggested that there is a limited time-window for this protein to be degraded in yeast ER and to start acquiring these O-mannosylations. It is not very clear from these experiments why the m∆gpαf would be associated with the translocon if it becomes stabilized in the lumen by O-mannosylation and not retrotranslocated. They proposed that Δgpαf might be engaged with the translocon for retrotranslocation while the O-mannosylation occurs, so this misfolded protein can be retained in the yeast ER. O-mannosylation was proposed to occur post-translationally in yeast ER by a slow and inefficient process (Nakatsukasa et al., 2004). O-mannosylation was suggested to maintain the proteins in a chaperone-independent, soluble form. In our in vitro retrotranslocation assay using mammalian microsomes, O-mannosylation of Δgpαf was
not detectable, so perhaps it is limited to yeast microsomes or occurred in a small, undetectable fraction of translocated ∆gpαf (Fig. 21).

A1PiZ is another widely used ERAD substrate that was investigated in this study (Fig. 28). This protein is the only soluble ERAD substrate beside ∆gpαf that has been demonstrated to retrotranslocate in an in vitro assay up to this day (Qu et al., 1996). The abnormally folded A1PiZ causes several serious diseases in humans. The wild type α-1-proteinase inhibitor (A1PiM, α1-antitrypsin inhibitor M-form) is synthesized by the liver and normally passes through the secretory pathway. A1PiM has three glycosylation sites and traffics through the ER very quickly. Its Z-form has a mutation E342K, which causes the salt bridge between K290 and E342 to be disrupted. This form accumulates in the ER lumen and is eventually degraded in the cytosol. This degradation was shown to be inhibited by 10 µM lactacystine and A1PiZ completely disappeared within 2-4 hours without proteasome inhibitors in vivo (Qu et al., 1996). A cell-free system developed by this research group used proteasome-treated reticulocyte lysate for in vitro translation of A1PiZ mRNA and translocation into KRM. The microsomes were sedimentsed and resuspended it into reticulocyte lysate without proteasome inhibitors. The reaction was run up to 16 hours while aliquots were removed about every hour, run on an SDS-PAGE gel, and quantified. This system supported the degradation on A1PiZ, while A1PiM remained stable. This approach was used in this dissertation (Fig. 28). When A1PiZ mRNA was translated in wheat germ extract, neither degradation nor retrotranslocation from KRM occurred. The KRM were sedimentsed and resuspended in reticulocyte lysate cytosol; aliquots were removed then during an hour incubation period (Fig. 27). It
was demonstrated that A1PiZ could be retrotranslocated in a reticulocyte cell-free extract (Fig. 28; lanes 1-6), because the amount of A1PiZ decreased over time in an Adenosine 5'-triphosphate (ATP)- and cytosol-dependent process (Fig. 28; lanes 7-15). In this respect, the degradation of ∆gpαf was very similar to the degradation of A1PiZ, but ∆gpαf was degraded in a faster rate.

The next task was to examine whether the in vitro translated and translocated ∆gpαf-BOF retrotranslocation was detectable spectroscopically. Some εBOF\textsuperscript{Lys} was incorporated into ∆gpαf, and the microsomes were purified by gel-filtration before the spectroscopic measurements. The results showed that the antibody in the cytosol quenched BOF when the in vitro translated ∆gpαf-BOF was transported from the ER lumen to the cytosol. There was a 10% decrease in the fluorescence intensity of the sample containing cytosol and EGS compared to the no cytosol sample over a 500 second time period. These preliminary results confirmed that ∆gpαf-BOF was retrotranslocated back to the cytosol, although the conditions to maximize the rate still needed to be optimized by future experiments (Fig. 29).

These in vitro experiments revealed that the dependence on ATP and cytosol for the retrotranslocation of ERAD substrates is universal, but may involve different individual cytosolic and lumenal components for various ERAD substrates. The identification of these factors/proteins in the ER lumen, membrane and cytosol was the goal of this project.
CHAPTER IV

CYTOSOLIC REQUIREMENTS FOR

THE RETROTRANSLOCATION OF A FLUORESCENCE-LABLED ERAD

SUBSTRATE*

Background and rationale

ERAD quality control machinery must distinguish misfolded proteins in the ER lumen from proteins that are on the way to folding properly, and ERAD substrates must be targeted to a retrotranslocation site in the ER membrane, transported through the membrane, and directed to sites of proteolysis (Johnson and Haigh, 2000). Proteins in and on both sides of the ER membrane are thus essential to work in concert to complete this procedurally difficult task. Since ERAD was first identified (Hampton et al., 1996; Hiller et al., 1996; McCracken and Brodsky, 1996; Sommer and Jentsch, 1993; Ward et al., 1995), a number of proteins have been shown to be involved directly or indirectly in ERAD. While the specific roles of some proteins have been determined, the functions of others are either unknown or controversial. The confusing and sometimes contradictory data about the involvement of specific proteins in retrotranslocation result in part from limitations in the experimental approaches currently available to examine this process, especially in mammalian systems. First, in most in vivo and in vitro studies, ERAD

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substrates are synthesized in the cytoplasm and co-translationally translocated into the ER lumen concurrent with ongoing retrotranslocation and degradation. Thus, approaches to study retrotranslocation are complicated by the inability to discriminate between substrates on their way into the ER lumen and those on their way out. Even the presence of a lumenal or cytosolic-specific covalent modification (e.g., signal sequence cleavage, glycosylation, ubiquitinylation) cannot distinguish between proteins that were completely translocated and those that were trapped at some stage prior to complete translocation. These realities interfere with accurate quantification of the extent of retrotranslocation. Second, the kinetics of retrotranslocation are difficult to quantify accurately, both for the above reasons and because retrotranslocation cannot be synchronized in most experiments. Since translocation and retrotranslocation proceed simultaneously both in vivo and in vitro at unknown and perhaps variable rates, there is currently no way to prepare samples in which all substrates are in the same state at a specific time. Hence, synchronous initiation of retrotranslocation for all substrates in a sample has been impossible. Third, the role of a particular protein in retrotranslocation is difficult to ascertain in many approaches. For example, the mutation of a particular gene may alter in vivo retrotranslocation activity, but it is difficult to know whether the observed change in activity is a primary or secondary effect. Interpretations are further complicated if the loss of a specific protein is obscured by the presence of another protein that is partially or wholly redundant.
**Experimental design**

To overcome these limitations, a mammalian in vitro system was developed that allowed us to monitor retrotranslocation synchronously and in real time in the absence of concurrent translocation (Fig. 30). Fluorescent-labeled ERAD substrate molecules and a

**Figure 30.** Reconstitution of canine pancreatic microsomes with various soluble lumenal contents. KRM (i, gray) release their soluble lumenal contents when subjected to high pH (ii). These open microsomes are purified from the soluble contents (ii, pink, orange, and purple, blue) by sedimentation. These microsomes are resuspended in a solution containing a BOF-labeled ERAD substrate with purified soluble lumenal components (all, some or none) and resealed by lowering the pH to 7.5 (iii). The microsomes with lumenal content (RRMs) or without any lumenal content (XRM) are resealed in the proper orientation and are fully functional. RRM are further purified by gel-filtration to remove any non-encapsulated proteins and directly used for fluorescence experiments.
known selection of luminal components is encapsulated inside ER microsomes using the procedures summarized in Fig. 30. The luminal contents of ER microsomes were extracted by exposure to pH 9.5-10.0 and separated from membranes by sedimentation (Alder et al., 2005; Bulleid and Freedman, 1988; Haigh and Johnson, 2002; Hamman et al., 1998; Nicchitta and Blobel, 1993). After the membranes were resuspended in a solution containing the components to be encapsulated (e.g., Δgpαf-BOF, ATP, BiP), the solvent pH was reduced to 7.5 to re-seal the microsomes in their original orientation (lumen inside) without any detectable loss of ribosome-binding activity. Following gel filtration to separate the microsomes from any nonencapsulated material, the reconstituted rough microsomes (RRMs) had a defined luminal content. When such microsomes are added to a solution containing a known selection of cytosolic components, the resultant sample is well-defined biochemically, with the desired molecular species on each side of the ER membrane. The retrotranslocation of fluorescent-labeled substrates from the luminal to the cytosolic side of the membrane is detected because the cytosol also contains fluorescence-quenching agents (Fig. 31). The rate of substrate retrotranslocation is therefore monitored directly in real time by the reduction in sample emission intensity as the dyes that are covalently attached to the substrate move through the ER membrane and are exposed to the quenchers. As all substrate molecules are initially encapsulated inside microsomes and retrotranslocation is initiated by increasing the temperature, substrate retrotranslocation is synchronized and reproducible. Moreover, by varying the luminal and cytosolic components in
Figure 31. Basis of fluorescence-detected retrotranslocation. (A) (i) RRM s (gray) containing Δgpαf-BOF (cyan with yellow dye), ATP, and a selection of lumenal proteins (?) = all, some or none) are mixed on ice with ATP, αBOF (anti-BOF antibodies; red), and some selection of cytosolic proteins (?) = all, some, or none. (ii) Retrotranslocation is initiated at t₀ by incubating samples at 30°C. (iii) Retrotranslocation of Δgpαf-BOF from the inside to the outside of the microsomes allows αBOF to bind the BOF dye and quench its emission intensity. (iv) A direct real-time measure of the rate and extent of Δgpαf-BOF retrotranslocation is therefore given by the time-dependent decrease in BOF intensity as more Δgpαf-BOF molecules are exposed to αBOF.

Parallel samples, the requirement for individual factors during retrotranslocation may be revealed by even subtle changes in the kinetics of substrate retrotranslocation.

The ERAD substrate used in this study was Δgpαf, a non-glycosylated derivative of the yeast pro-α-factor mating pheromone (Fig. 32). Because both yeast (Caplan et al., 1991; McCracken and Brodsky, 1996) and mammals (Su et al., 1993) recognize and degrade Δgpαf, it is a valid model ERAD substrate. Furthermore, Δgpαf is soluble, is not ubiquitinylated, and lacks disulfide bonds, carbohydrates, and transmembrane sequences. Since these structural features may complicate retrotranslocation and require additional or different mechanisms and/or components, Δgpαf was used to establish the minimal requirements for retrotranslocation and characterize the system with in this dissertation.
Figure 32. The structure of the wild type and mutant pro-α-factor. The wild type yeast prepro-α-factor mating pheromone (ppαf) is shown in the upper panel. The three potential glycosylation sites are shown on the wild-type protein. The mutant, unglycosylated (Δgpαf) protein without the signal sequence, which is an ERAD substrate, is shown in the bottom panel. A single cysteine was substituted at either position 25 or 165 if the protein and then covalently modified by the attachment of a fluorescent dye as shown with a yellow star (either Y165C or T25C). Data presented in this dissertation were obtained with the construct that had the dye attached to the C-terminal end (Y165C), unless specified otherwise.

Δgpαf-BOF and microsomes with defined lumenal contents

One advantage of the spectroscopic approach is that a substrate from a single prep of Δgpαf-BOF can be encapsulated and compared with parallel samples that have different combinations of lumenal and/or cytosolic components. This approach also eliminates substrate heterogeneity (Fig. 30). A recombinant Δgpαf derivative was over-expressed and purified with a C-terminal hexameric histidine tag and four single-site mutations: N23Q, N57Q, and N67Q to prevent glycosylation, and Y165C for dye attachment (Fig.
Since no targeting to the translocon was required, no signal sequence was present.

This Δgpαf derivative was then covalently modified with BOF and gel filtered to
remove unreacted dye, thereby yielding Δgpαf-BOF. To assess whether Δgpαf-BOF
adsorbs to the outer surface of RRM after the reconstitution procedures, microsomes
were subjected to mock encapsulation procedures in the absence of Δgpαf-BOF and then
incubated with Δgpαf-BOF to allow adsorption. After gel filtration, the BOF intensity of
mock RRM was ~10% of that seen with RRM that contained Δgpαf-BOF. Since any
cytosol-exposed Δgpαf-BOF was quenched before retrotranslocation was initiated, the
molecules absorbed to the outer microsomal surface did not contribute to the spectral
changes shown below. An independent, although less accurate, determination of Δgpαf-
BOF absorbed to the outside was carried out by proteinase-K (PK) digestion (Fig. 33).
The Δgpαf-BOF was visualized by fluorescence and quantified. PK only digested
Δgpαf-BOF absorbed to the outside microsomal surface, and this was about 10% of the
original signal after quantification (compare Fig. 33; lanes 1 and 2). The Δgpαf-BOF
almost completely disappeared after addition of TX-100 to solubilized the membranes.
These experiments confirmed that the majority of Δgpαf-BOF was within the RRM, but
one must emphasize that proteinase experiments are clearly not as accurate as
determination of the amount of Δgpαf-BOF outside of the microsomes by antibody
quenching (only ~5% of the initial signal was quenched after addition αBOF to the
RRM with Δgpαf-BOF encapsulated, but αBOF quenches BOF conjugated to a protein
by 50% efficiency) or by mock reactions (~10%).
Figure 33. Fluorescence-detected protease protection assay with reconstituted Δgpαf-BOF. Δgpαf-BOF was reconstituted into canine pancreatic microsomes and separated by gel filtration over Sepharose CL-2B from any non-encapsulated Δgpαf. Proteinase K (0.1 mg/ml final concentration) or proteinase K and TX-100 [(v/v) 1% final concentration] were added to purified, reconstituted vesicles and samples were incubated on ice for 30 min. Reactions were terminated by addition of 1 mM PMSF. Samples were analyzed by SDS-PAGE and the bands were quantified using the BioRad phosphorimager by BODIPY fluorescence.

**Fluorescence-detected retrotranslocation**

In initial retrotranslocation experiments, microsomes contained Δgpαf-BOF, ATP, and a full set of lumenal proteins that had been extracted, concentrated, and then reconstituted back into RRM (complete RRM). To obtain a full set of cytosolic proteins, rabbit reticulocyte lysate was first chromatographed to remove hemoglobin because its presence interfered with fluorescence measurements. The resulting hemoglobin free lysate, here termed “cytosol or cytosol-Hb” (Figs. 23 and 24), was diluted ~4-fold from the original lysate, but still supported retrotranslocation (Fig. 21)
Figure 34. The retrotranslocation of Δgpαf-BOF is spectroscopically detectable. Complete RRMs containing Δgpαf-BOF with total luminal proteins, and ATP were purified by Sepharose CL-2B and resuspended in cytosol, an EGS and +/- αBOF. Sample fluorescence intensity was monitored with mixing in the fluorometer for 2000 seconds. A time-dependent decrease in fluorescence intensity can be observed over this time-period, which is due to the quenching of the BOF dye when Δgpαf-BOF is transported out of the microsomes and into cytosol containing ATP.

and was used in all fluorescence experiments. A solution containing cytosol and ATP is designated “complete cytosol”. The concentrations of ATP, cytosol, and luminal proteins required for maximal retrotranslocation were determined and used throughout this study. To detect substrate retrotranslocation, BOF-specific antibodies (αBOF) were added to the cytosol. Since no retrotranslocation occurs at 0°C (Fig.53), RRMs were mixed with the cytosolic components and αBOF on ice before retrotranslocation was initiated by raising the temperature to 30°C. Besides allowing complete mixing, the 0°C incubation allowed αBOF to quench any residual non-encapsulated Δgpαf-BOF before the reaction was initiated (t₀). Since αBOF binding to BOF strongly quenches its
emission, any retrotranslocation of lumenal Δgpαf-BOF into the cytosol should result in a significant reduction in sample emission intensity (Fig. 34). The magnitude of the spectral change depends on the initial state of BOF in the sample because BOF emission is partially quenched in the full-length polypeptide, and proteolysis of Δgpαf-BOF causes a 65% increase in BOF intensity (see later). To focus solely on αBOF-dependent intensity changes, parallel samples with or without αBOF were examined in each experiment. The magnitude of the αBOF-dependent intensity change was then given by the ratio of intensities with and without αBOF (F = F_{+αBOF} / F_{−αBOF}). Only the αBOF-dependent intensity is shown here. When complete RRMss were added to complete cytosol, a substantial αBOF-dependent decrease in BOF intensity was observed (Fig. 34). Maximal αBOF-dependent quenching averaged 46 ± 1% of the initial intensity after 2000 sec (Fig. 34).

**Cytosol- and ATP-dependent substrate retrotranslocation**

When complete RRMss were incubated in the absence of cytosolic proteins, the initial sample intensity was reduced by 9 ± 1% after 2000 sec (Fig. 35; red trace). When apyrase, an enzyme that hydrolyzes ATP, was included in both the lumenal and cytosolic solutions, the rate and extent of Δgpαf-BOF quenching were reduced to the same extent (10 ± 3%) (Fig. 36; green trace).
Figure 35. Cytosol is required for the retrotranslocation of Δgpαf-BOF. Complete RRM s encapsulating Δgpαf-BOF and all soluble luminal proteins were incubated in the presence (blue) or absence (red) of cytosol, an EGS and +/- αBOF. Retrotranslocation was monitored in the fluorometer for 2000 seconds.

Figure 36. ATP is required for the retrotranslocation of Δgpαf-BOF. The αBOF-dependent quenching is shown for complete RRM s encapsulating Δgpαf-BOF upon incubation in complete cytosol (blue; n = 48), ATP in the absence of cytosolic proteins (red; n = 21), and cytosol lacking ATP (green; n = 7; 30 units/ml apyrase was included in both the lumen and the cytosol).
Thus, both cytosolic proteins and ATP are required for Δgpαf-BOF retrotranslocation, as observed in yeast systems (McCracken and Brodsky, 1996; Pilon et al., 1997; Lee et al., 2004).

**Origin of background fluorescence quenching**

The fluorescence approach utilized here measures the kinetics of ERAD substrate exposure to the cytosol continuously at a time resolution much higher than that achievable by other methods.

![Figure 37. Cytosol-dependent retrotranslocation of Δgpαf-BOF during a longer time period.](image)

Complete RRM s containing Δgpαf-BOF were incubated for a longer time with complete cytosol (red) or without cytosolic proteins (blue).

There was a small steady decrease in Δgpαf-BOF intensity observed in the absence of either cytosol or ATP (Figs. 35 and 37). Is the slow, steady increase in αBOF-dependent quenching in the absence of cytosolic proteins or ATP caused by encapsulated material leaking from the microsomes?
Figure 38. Retrotranslocation-independent exposure of BOF-labeled polypeptides to cytosol. (A) Complete RRMs containing ATP, total lumenal proteins, and either glutathione-BOF (red), PDI-BOF (blue), or BiP-BOF (orange) were incubated in complete cytosol. RRMs containing glutathione-BOF were also incubated in the absence of cytosol (cyan). \( n = 3 \) to 5 independent experiments. The small steady decrease in \( \Delta gpo\alpha f-\)BOF intensity observed in the absence of either cytosol or ATP (Fig. 36) was nearly identical to the decrease observed with RRMs containing BODIPY-labeled glutathione, heavy chain binding protein (BiP) or protein disulfide isomerase (PDI). Since BiP and PDI are ER resident proteins and are not retrotranslocation substrates, this \( \alpha \)BOF-dependent quenching appears unrelated to retrotranslocation.

RRMs were reconstituted with ATP, total lumenal proteins, and BOF-labeled glutathione to determine if the release of a small molecule occurs spontaneously from the RRMs over time. Other RRMs were prepared with ATP and either BOF-labeled BiP or BOF-labeled PDI because these abundant ER lumenal proteins should not be substrates for retrotranslocation (Fig. 38). When incubated in full cytosol, the rates and extents of quenching, and hence BOF exposure to \( \alpha \)BOF in the cytosol, were the same for both large (BiP-BOF, PDI-BOF) and small (glutathione-BOF) encapsulated molecules. Thus, any openings in the RRMs had to be large enough to release PDI and BiP at the same rate as glutathione. Yet we showed previously that microsomal
membranes are impermeable to iodide ions for more than 4 hours at 4°C (Crowley et al., 1994). Thus, holes large enough to release PDI or BiP from microsomes are unlikely, and the slow increase in αBOF-dependent quenching in the absence of retrotranslocation apparently does not occur due to either glutathione-BOF or Δgpαf-BOF leakage from RRM. Instead, this signal loss most likely results from a low constant rate of RRM breakage at 30°C that simultaneously exposes glutathione-BOF and the larger Δgpαf-BOF, PDI-BOF, and BiP-BOF to αBOF. Whatever its origin, this αBOF-dependent emission intensity decrease appears to constitute a “background” signal change because it is observed under conditions in which retrotranslocation does not occur (compare Fig. 36 “no cytosol” and “no ATP” traces with Fig. 38). Moreover, after very long time periods (~50 min), the rate of quenching was the same regardless of whether or not cytosol was present (Fig. 37). Thus, the quenching due to retrotranslocation was complete within ~50 min under our conditions in the sample containing cytosolic proteins, and the net intensity reached a plateau by 50 min (Fig. 37). Therefore the αBOF-dependent quenching observed with samples lacking cytosol was routinely subtracted to accurately portray the retrotranslocation-dependent fluorescence change (Fig. 39).
Complete RRMs containing ∆gpαf-BOF and ATP were incubated for an extended time with or without cytosol, and the net αBOF-dependent quenching (red; n = 3) was obtained by subtracting the non-cytosol αBOF-dependent quenching from the complete cytosol αBOF-dependent quenching (Fig. 37). The non-cytosol dependent quenching was also subtracted from itself to yield the expected flat line (blue). Subtracting the background from the observed signal change yielded this net retrotranslocation-dependent fluorescence change. ∆gpαf-BOF retrotranslocation was complete when the spectral change reached a plateau, which occurred after 3000 sec under our conditions.

Detection of ∆gpαf-BOF in the cytosol after retrotranslocation

Direct biochemical evidence for lumenal ∆gpαf-BOF transport into the cytosol was provided by incubating complete RRMs with ATP and epoxomicin-treated cytosol in the absence of αBOF (Fig. 40), removing the microsomes by sedimentation, and quantifying the amount of ∆gpαf-BOF fluorescence in the supernatant. On average (n = 3), 30% of full-length ∆gpαf-BOF was found in the supernatant under conditions when the proteasome was inhibited by epoxomicin (Fig. 40, lane 4). Much less ∆gpαf-BOF was found in the supernatant when proteasomes were active (Fig. 40, lane 2), and none was found when no retrotranslocation occurred (Fig. 40, lane 3).
Figure 40. **Fluorescence-detected accumulation of Δgpαf-BOF in the cytosol after retrotranslocation.** Complete RRMss containing Δgpαf-BOF were incubated (30°C, 30 min) with complete (lane 2), without cytosol (lane 3), or complete cytosol inhibited with 60 µM epoxomicin (lane 4); an equivalent amount of RRMss was loaded directly into lane 1. After microsomes and encapsulated Δgpαf-BOF were removed by sedimentation, the amount of full-length Δgpαf-BOF in each supernatant was determined by SDS-PAGE and imager-detected BOF fluorescence.

The AAA protease, p97, with its two co-chaperones (Npl4 and Ufd1) do not stimulate Δgpαf retrotranslocation

The ATPase complex containing p97 (VCP; Cdc48 in yeast), Ufd1 and Npl4 play a critical role in the retrotranslocation of several ERAD substrates (Bays and Hampton, 2002; Carlson et al., 2006; Tsai et al., 2002). To show that there
Figure 41. Western blot shows no detectable residual p97 bound to RRM5s after the high pH treatment. Isolated p97 (VCP) and the p97/Npl4/Ufd1 complex each have ATPase activity, and the p97/Npl4/Ufd1 complex binds to polyubiquitin chains (G. DeMartino, personal communications). These p97 were incubated with 10 eq/µl KRM5s or RRM5s, or these microsomes were incubated with only buffer at 4 °C for 20 minutes and then 30 °C for 10 minutes. The resulting microsomes were sedimented and the pellet was resuspended in SDS-PAGE running buffer. The bands were detected by chemifluorescence on BioRad phophorimager with antibodies to p97.

was not any residual p97 present on the ER membrane, western blotting was done (Fig. 41). While the KRM5s contained residual p97 (although some might have sedimented independently of the microsomes due to its size), the Western blot shows no detectable residual p97 bound to RRM5s after the high pH treatment. Isolated p97 (VCP) and the
Figure 42. Retrotranslocation of Δgpαf-BOF does not depend on p97-Npl4-Ufd1. Complete RRM
containing Δgpαf-BOF were incubated with ATP and p97, Ufd1, and Npl4 (red; n = 4), full cytosol (blue),
or no cytosol (cyan). The longer time-scale graph is shown on the left and the shorter time-scale is on the
right.

p97-Npl4-Ufd1 complex each have ATPase activity, and the p97-Npl4-Ufd1 complex
binds to polyubiquitin chains (DeMartino, personal communication).

To assess the involvement of this complex in Δgpαf retrotranslocation, complete
RRMs were incubated with purified p97, Ufd1, Npl4, and ATP, and no increase in
αBOF-dependent quenching over background was observed (Fig. 42).

A different approach to confirm the involvement of p97 in retrotranslocation was
utilized when cytosol was incubated with excess antibodies to p97 (αp97) prior to
addition to the ERAD assay. The quenching rates of the untreated and did not change
compared to the full cytosol sample when excess αp97 was added (Fig. 43), thereby
showing that a p97-αp97 complex prior to exposure to microsomal membranes did not
alter the rates of retrotranslocation. Thus, Δgpαf retrotranslocation is independent of p97 and its cofactors in this system.

**Cytosolic requirements for Δgpαf-BOF retrotranslocation can be replaced with purified 26S proteasomes**

In yeast, 26S proteasomes stimulate Δgpαf retrotranslocation (Lee et al., 2004) and possibly that of other substrates (e.g., (Chillarón and Haas, 2000; Mayer et al., 1998; Walter et al., 2001). When complete RRM s were incubated with ATP and purified bovine 26S proteasomes, the maximal rates and extents of αBOF-dependent quenching were nearly the same for pure 26S proteasomes and for total cytosolic proteins (Fig. 44).
Figure 44. Cytosolic requirements for the retrotranslocation of ∆gpαf-BOF can be substituted by purified 26S proteasomes. Complete RMs containing ∆gpαf-BOF were incubated with ATP and 26S proteasomes (red; n = 17), complete cytosol (cyan), or without cytosol (blue). The longer time-scale graph is shown on the left and the shorter time-scale is on the right.

PA700, the 19S regulatory particle (RP) of the 26S proteasome, stimulates ∆gpαf retrotranslocation

To determine whether PA700 alone could promote retrotranslocation, complete RMs were incubated with ATP and purified bovine PA700. Since an increase in αBOF-dependent quenching was observed (Fig. 45), PA700 stimulated ATP-dependent ∆gpαf retrotranslocation. Moreover, the ATPase activity of PA700 was required because pre-treating PA700 with N-ethylmaleimide (NEM; (DeMartino et al., 1994) essentially blocked retrotranslocation (Fig. 46). Continuous monitoring of ∆gpαf-BOF intensity revealed that the initial quenching rates were similar for samples containing either purified 26S or purified 19S RPs (Figs. 44 and 45, right panel), but the total 19S-dependent increase in quenching was much less than the total 26S-dependent increase (Fig. 45).
Figure 45. PA700, the 19S proteasomal regulatory particle, stimulates Δgpαf retrotranslocation. Complete RRMs containing Δgpαf-BOF were incubated with ATP and PA700 regulatory particles (RPs) (red; n = 10), without cytosol (blue), or complete cytosol (cyan). The longer time-scale graph is shown on the left and the shorter time-scale is on the right.

Figure 46. Δgpαf retrotranslocation does not occur in the presence of NEM-treated 19S RP. Complete RRMs containing Δgpαf-BOF were incubated with ATP and NEM-treated 19S proteasomes (red; n = 4), 19S RPs (green; n = 10), no cytosol (blue), or complete cytosol (cyan).
The cause of the differential quenching between 26S- and 19S-containing samples was revealed by incubating complete RRMs with ATP and 26S that had been pre-treated with the proteasome inhibitors epoxomicin or lactacystine (Gaczynska and Osmulski, 2005). The rates and extents of αBOF-dependent quenching were nearly the same for samples containing PA700 (Fig. 45), epoxomicin-treated 26S (Fig. 47), epoxomicin-treated (Fig. 48) or lactacystin-treated 26S cytosol (Fig. 49). Hence, the 26S protease activity was responsible for the different quenching profiles observed with 19S and 26S. This conclusion was confirmed by measuring the BOF intensity of intact and proteolyzed Δgpαf-BOF and its sensitivity to αBOF (data not shown).

Figure 47. The rate and extent of Δgpαf retrotranslocation are similar in the presence of epoxomicin-treated 26S proteasome and 19S RP. Complete RRMs containing Δgpαf-BOF were incubated with ATP and epoxomicin-treated 26S proteasomes (red; n = 4), 19S RPs (green; n = 10), without cytosol (blue), or complete cytosol (cyan). The longer time-scale graph is shown on the left and the shorter time-scale is on the right.
Since the spectral change observed in 26S-containing samples includes contributions from retrotranslocation and degradation, the kinetics of retrotranslocation are best quantified using spectral changes that are independent of substrate digestion. Thus, the rate of Δgpαf-BOF exposure to the cytosol under our conditions is more accurately given by the $t_{1/2}$ (the time required to reach 50% of maximal quenching) in 19S-containing samples (2.2 min; Fig. 45) than by the $t_{1/2}$ in 26S-containing samples (8.3 min; Fig. 44). These data also reveal that the maximal initial rate of Δgpαf-BOF retrotranslocation can be achieved solely with ATP and PA700 (Fig. 47).

Figure 48. The rate and extent of Δgpαf retrotranslocation are similar in the presence of epoxomicin-treated cytosol and 19S RP. Complete RRMss containing Δgpαf-BOF were incubated with ATP and epoxomicin-treated cytosol (red; $n = 4$), 19S RPs (green; $n = 10$), without cytosol (blue), or complete cytosol (cyan). The longer time-scale graph is shown on the left and the shorter time-scale is on the right.
Figure 49. The rate and extent of Δgpαf retrotranslocation are similar in the presence of lactacystin-treated 26S and 19S RP. Complete RRMks containing Δgpαf-BOF were incubated with ATP and lactacystin-treated cytosol (red; n = 3), 19S RPs (cyan; n = 10), without cytosol (blue), or complete cytosol (black). The longer time-scale graph is shown on the left and the shorter time-scale is on the right.

Figure 50. Fluorescence-detected proteolysis of Δgpαf-BOF after retrotranslocation from RRMS. Complete RRMks containing Δgpαf-BOF were incubated at 30°C in either rabbit reticulocyte lysate (cytosol; lanes 1-3), or rabbit reticulocyte lysate cytosol without hemoglobin (cytosol–Hb; lanes 4-6), or rabbit reticulocyte lysate cytosol without hemoglobin preincubated (0°C, 30 min) with 50 μM lactacystin (lanes 7-9). Equivalent aliquots of the total sample were removed at the times indicated and analyzed by SDS-PAGE. Full-length Δgpαf-BOF was detected by BOF emission intensity.
Fluorescence and αBOF quenching of intact and proteolyzed Δgpαf-BOF

Retrotranslocated Δgpαf-BOF was degraded when complete RRM3s were incubated in cytosol at 30°C (Fig. 50, lanes 4-6). Since this proteolysis was prevented by preincubating the cytosol with the proteasome-specific inhibitor lactacystin (Fig. 50, lanes 7-9), Δgpαf-BOF was degraded solely or primarily by the 26S proteasome. Biophysical and biochemical experiments also showed that full-length non-ubiquitinated Δgpαf-BOF was degraded by purified 26S proteasomes, but not by purified epoxomicin-inhibited 26S proteasomes (Figs. 51 and 52). The reduction in BOF anisotropy (r) results

![Figure 51. Fluorescence-detected proteolysis of Δgpαf-BOF. The average (n = 3) anisotropy (r) of 0.5 μM purified Δgpαf-BOF was monitored as a function of time at 30°C in samples containing 20 μg/ml purified 26S proteasomes that had not (red) or had (blue) been preincubated (0°C, 30 min) with 60 μM epoxomicin.](image-url)
Figure 52. 26S proteasomes degrade Δgpαf-BOF even though it lacks ubiquitin. Biochemical evidence for the degradation of free (non-encapsulated) Δgpαf-BOF by purified 26S proteasomes is shown above. Purified Δgpαf-BOF was incubated with either purified 20 µg/ml 26S proteasomes with or without 50 µM epoxomicin pre-treatment (lanes 2-3), 0.3 mg/ml cytosol with or without 60 µM epoxomicin pre-treatment (lanes 4-5), or no cytosol and 26S proteasomes (Lane 1).

from an increase in the dye’s rate of rotation as the polypeptide to which the dye is attached is reduced in size by proteolysis. The amount of Δgpαf-BOF was significantly decreased when free Δgpαf-BOF was incubated with purified 26S proteasomes or cytosol in the absence of epoxomicin (Fig. 52). Δgpαf-BOF was detected on an SDS-PAGE gel by BOF-fluorescence.

When Δgpαf-BOF was completely degraded by the 26S proteasome (shown by a reduction in BOF anisotropy from 0.13 to 0.04), BOF emission intensity was increased by 65%. Thus, BOF emission is quenched by 40% in intact Δgpαf. BOF emission is quenched upon binding to BOF-specific antibodies, and the intensity of the quenched
BOF is the same whether BOF is attached to intact or degraded Δgpαf. Thus, the magnitude of antibody quenching depends upon the state of the Δgpαf polypeptide. When αBOF binds to intact Δgpαf-BOF, BOF intensity is reduced by 56%. When excess αBOF is added to proteolyzed Δgpαf-BOF, BOF intensity was reduced by 72%. Hence, the αBOF-dependent quenching ranges between 56% and 72% when Δgpαf-BOF is actively degraded in a sample. Since αBOF binding to either intact or degraded Δgpαf-BOF is complete by the time the sample has been mixed, no detectable delay for αBOF binding to Δgpαf-BOF is observed, and no correction of the kinetic data is necessary. This difference explains why the observed spectral change was greater (net $F/F_0$ was smaller) in 26S than in 19S samples (Fig. 45), since the fluorescence quenching by αBOF was greater in samples in which Δgpαf-BOF was degraded.

Thus, there were four components to the spectral signal in our experiments: the Δgpαf-BOF molecules that were retrotranslocated and thereby exposed to αBOF; the Δgpαf-BOF molecules that were exposed to αBOF because of background microsome release during the incubation at 30°C; the Δgpαf-BOF molecules that were degraded in the cytosol; and the few Δgpαf-BOF molecules that were adsorbed to the outer surface of the microsomes after gel filtration (<10%). Clearly, it is essential to identify, quantify, and thoroughly characterize the biochemical origin of any spectral changes before interpreting them (Johnson, 2005b). The fraction of Δgpαf-BOF that is retrotranslocated from RRM in our samples can be calculated using the above intensities for different species. Assuming that 10% of the Δgpαf-BOF is adsorbed to the outer surface of the
microsomes and that 10% of the remaining ∆gpαf-BOF (= 9%) is exposed to the cytosol by the background release of contents from microsomes (Fig. 35), a total intensity decrease of 46% (Fig. 34) in complete RRMs (when all lumenal proteins are present) with full cytosol under these conditions would result from 36% of the originally encapsulated ∆gpαf-BOF being retrotranslocated from the lumen to the cytosol and then digested within 2000 sec. As noted in the text, this magnitude of retrotranslocation is similar to the fraction of ∆gpαf-BOF found in the cytosol after the microsomes have been sedimented (Fig. 40).

**Temperature effects**

Complete RRMs were incubated in parallel with or without cytosolic proteins at different temperatures to evaluate the effect of temperature on retrotranslocation (Fig. 53, left). Since the cytosol-free background quenching was temperature-dependent (Fig. 53; broken lines, left), each cytosol-free signal was subtracted from the corresponding signal in the presence of cytosol (Fig. 53; solid lines, left) to yield the net αBOF-dependent quenching at each temperature (Fig. 53; right). These data revealed that both the rate and extent of mammalian retrotranslocation are temperature dependent under our conditions. There was not any retrotranslocation detected at 4°C during the one-hour incubation period. Hence, no retrotranslocation occurs while our samples are on ice prior to raising their temperature at t₀ to initiate retrotranslocation. The net αBOF-dependent quenching (retrotranslocation) was essentially the same at 37°C and 30°C (Fig. 53). Thus, our experiments were done at 30°C because the non-retrotranslocation related
release of Δgpαf-BOF by apparent microsomal rupture (see above) was greater at 37°C than at 30°C.

Discussion

The real-time continuous detection of Δgpαf-BOF retrotranslocation and its dependence on various components provides a unique perspective on the mechanisms and roles of individual proteins during retrotranslocation in mammals. This study of the cytosolic requirements revealed that Δgpαf-BOF retrotranslocation occurred rapidly and was complete within minutes. Δgpαf-BOF retrotranslocation required both ATP and the proteasome. When purified 26S proteasomes or purified PA700 replaced the cytosol, Δgpαf-BOF retrotranslocation occurred at the same rate as with total cytosolic proteins.
The degradation of Δgpαf- BOF was ubiquitin independent. The AAA protease p97 with its cofactor did not play a role in Δgpαf- BOF retrotranslocation.

The novel fluorescence approach developed in this research project characterizes retrotranslocation with unprecedented time resolution. ERAD substrate movement from the lumen to the cytosol is detected directly and in real time, thereby allowing both the kinetics and extent of retrotranslocation to be reproducibly quantified and compared. Since this spectral approach is non-destructive, substrate environment can be monitored continuously. Most important, this approach examines substrate retrotranslocation under native conditions. Since membranes and macromolecular complexes are intact throughout, the functional effects of even weak or transient interactions or assemblies can be detected without being disrupted by the detergent required for some analyses. Furthermore, since different aspects of the fluorescence signal can be monitored [e.g., intensity, anisotropy, lifetime, fluorescence resonance energy transfer or FRET, accessibility to quenchers (Johnson, 2005a; Johnson, 2005b), spectral changes can simultaneously monitor both retrotranslocation and other changes in substrate environment and conformation. The ability to resolve different intermediate states and interactions of the substrate allows mechanistic questions to be addressed at a higher resolution. For example, spectroscopically differentiating between Δgpαf-BOF exposure to and degradation in the cytosol (Fig. 48) shows that retrotranslocation precedes substrate proteolysis, as expected, and that the actual rate of retrotranslocation is best determined using fluorescence rather than proteolysis assays.
It is more than likely that the characterization of some other ERAD substrate may require other components or pathways for retrotranslocation than the Δgpαf-BOF examined here. For example, certain lumenal factors appear to be involved in the retrotranslocation of glycosylated ERAD substrates (Hebert et al., 2005). But the experimental approach described here can, in principle, be used with any soluble ERAD substrate or derivative. The biochemical reconstitution of samples provides great flexibility in examining the influence of specific soluble proteins or small molecules on either side of the membrane on the rate and extent of retrotranslocation. Not only are the samples homogeneous and well defined in terms of components and their concentrations, the substrates are in a uniform environment and conformation inside the lumen. Deleting a wild-type protein or replacing it with a purified mutant protein during reconstitution, as was done here, provides the same flexibility in a mammalian system that is achieved genetically in yeast.

The presence of p97, Npl4, and Ufd1 in the cytosol, either by themselves or added to total cytosolic proteins, did not stimulate Δgpαf retrotranslocation (Fig. 42). Since these proteins facilitate the retrotranslocation of most examined ubiquitinylated substrates (Bays and Hampton, 2002; Tsai et al., 2002) and Δgpαf is not ubiquitinylated, this result may be expected. But it shows, contrary to some suggestions, that p97 is not required for the retrotranslocation of every ERAD substrate (Carlson et al., 2005; Carlson et al., 2006; Kothe et al., 2005; Lee et al., 2004). Instead, PA700 is sufficient for maximal Δgpαf retrotranslocation (Fig. 45). Since ATP is also required (Fig. 36) and NEM-treated PA700 was defective in retrotranslocation (Fig. 46), it seems likely that AAA
ATPase components of PA700 are involved in powering substrate movement through the membrane via the chaperone-like properties of PA700 (Liu et al., 2006; Strickland et al., 2000). These findings also create a new set of questions. For example, what are the roles of individual PA700 subunits in retrotranslocation?
CHAPTER V
LUMENAL REQUIREMENTS FOR
THE RETROTTRANSLOCATION OF A FLUORESCENCE-LABELED ERAD
SUBSTRATE *

Lumenal proteins are required for maximal Δgpαf retrotranslocation

In order to determine whether lumenal proteins are essential for Δgpαf-BOF retrotranslocation, RRM were reconstituted with Δgpαf-BOF, ATP and various combinations of lumenal proteins. When RRM without any lumenal proteins were compared with complete RRM, the net αBOF-dependent quenching was substantially reduced (Fig. 54, red).

Thus, lumenal proteins are required for maximal Δgpαf-BOF retrotranslocation. However, some Δgpαf-BOF retrotranslocation occurred at a slow rate even in the absence of lumenal proteins.

In order to determine the amount of lumenal proteins that would maximize the retrotranslocation rate, an increasing amount of purified soluble lumenal proteins were encapsulated into RRM with Δgpαf-BOF and ATP before retrotranslocation was commenced. The lumenal protein concentration that gave maximal retrotranslocation was found to be 3.5 mg/ml (Fig. 55). Increasing the concentration of lumenal proteins to

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Figure 54. The retrotranslocation Δgpαf-BOF depends on lumenal components. RRM s containing Δgpαf-BOF and 2 mM ATP were reconstituted with the following components: total lumenal proteins (black); no lumenal proteins (red, n = 5). Each RRM was then incubated with complete cytosol at 30°C. For comparison, complete RRMs incubated in the absence of all cytosolic proteins are shown (blue). The net αBOF-dependent spectral changes are shown after subtraction of no cytosol background (Fig. 39).

7.0 mg/ml did not increase the rate or extent of Δgpαf-BOF retrotranslocation. Thus, the 3.5 mg/ml concentration of soluble lumenal proteins was used throughout this study.

Each successive preparation of soluble lumenal proteins was then titrated to ensure that it provided the same maximal rate of retrotranslocation.
Figure 55. Lumenal content required for maximal retrotranslocation rate. RRM containing Δgpαf-BOF and 2 mM ATP were reconstituted with an increasing amount of soluble lumenal proteins: total lumenal proteins used in the experiments (blue; 3.5 mg/ml); more lumenal proteins (orange; 7 mg/ml; n=2); fewer lumenal proteins (cyan and green; 1 mg/ml and 2 mg/ml respectively; n=2). Each RRM was then incubated with complete cytosol at 30°C. For comparison, complete RRM incubated in the absence of all lumenal proteins are shown (red). The net αBOF-dependent spectral changes are shown after subtraction of cytosol-free background (Fig. 39).

The retrotranslocation of Δgpαf-BOF does not depend on BiP

To assess whether individual lumenal proteins stimulate retrotranslocation, RRM containing only BiP, ATP and Δgpαf-BOF in the lumen were incubated in a complete set of cytosolic proteins. BOF quenching was higher with these RRM than with RRM lacking all lumenal proteins (Fig. 56; red), which showed that BiP stimulated Δgpαf-BOF retrotranslocation. However, the extent of BiP stimulation was much less than that
Figure 56. The retrotranslocation of Δgpd-BOF does not depend on BiP. RRM containing Δgpd-BOF and 2 mM ATP were reconstituted with the following components: total lumenal proteins (black); no lumenal proteins (cyan, n = 5); 10-20 µM BiP (red, n = 4). Each RRM was then incubated with complete cytosol at 30°C. For comparison, complete RRMs incubated in the absence of all cytosolic proteins are shown (blue). Net αBOF-dependent spectral changes are shown after subtraction of no cytosol background (Fig. 39).

seen with a full complement of lumenal proteins, and was not increased by increasing the BiP concentration further. The amount of BiP that was necessary to maximize the retrotranslocation rate was also determined by titration. Increasing amounts of purified BiP were encapsulated into RRM with ATP and Δgpd-BOF, and the concentration was chosen that did not increase the retrotranslocation rate any further (Fig. 57). The optimal BiP concentration was between 10-20 µM, since more BiP (Fig. 57; cyan) did not change the retrotranslocation rate significantly. In addition, fewer encapsulated BiP (2 µM and 5 µM; Fig. 57; green and red, respectively) did not significantly stimulate the
Figure 57. **BiP concentration required for maximal retrotranslocation.** RRs containing Δgpαf-BOF and 2 mM ATP were reconstituted with an increasing amount of BiP: total BiP used in the experiments (gray; 10-20 µM); more BiP (cyan; 20-30 µM; n=2); and less BiP (green and red; 2 µM and 5 µM respectively; n=2). Each RRM was then incubated with complete cytosol at 30°C. For comparison, complete RRMs incubated in the absence of all lumenal proteins are shown (black). The net αBOF-dependent spectral changes are shown after subtraction of the cytosol-free background (Fig. 39).

retrotranslocation of Δgpαf-BOF, since these microsomes had nearly the same rate as RRMs of retrotranslocation lacking all soluble luminal proteins (Fig. 57; black).

**The retrotranslocation of Δgpαf is stimulated by oxidized PDI**

In contrast to BiP when only PDI, ATP, and Δgpαf-BOF were reconstituted into RRMs, the maximal rate and extent of BOF quenching were essentially equivalent to those obtained with RRMs containing a full complement of luminal proteins (Fig. 58; red). Thus, only PDI is required in the ER lumen for maximal Δgpαf-BOF retrotranslocation.
Figure 58. The lumenal requirements for $\Delta$gp$\alpha$F retrotranslocation can be substituted by purified PDI. RRMs containing $\Delta$gp$\alpha$F-BOF and 2 mM ATP were reconstituted with the following components: total lumenal proteins (black); no lumenal proteins (cyan, $n = 5$); untreated PDI (red, $n = 7$). Each RRM was then incubated with complete cytosol at 30°C. For comparison, complete RRMs incubated in the absence of all cytosolic proteins are shown (blue). The net $\alpha$BOF-dependent spectral changes are shown after subtraction of the cytosol-free background (Fig. 39).

Interestingly, the PDI used in these experiments (Fig. 58) was not treated with any oxidized or reduced glutathione (GSSG or GSH, respectively) before encapsulation into the RRMs. However, PDI becomes oxidized in aqueous solution exposed to air (Neil Bulleid, personal communication). Evidence that PDI is air-oxidized is also shown by the fact that untreated (air-oxidized) and GSSG-oxidized PDI had similar affinities for $\Delta$gp$\alpha$F-BOF. The PDI concentration required to obtain maximal $\Delta$gp$\alpha$F-BOF retrotranslocation rate was determined by titration of an increasing amount of PDI into RRMs (Fig. 59). The optimal concentration was between 10-20 $\mu$M (Fig. 59; pink), but this optimum also depended on the individual PDI preparation. The addition of additional PDI (20-30 $\mu$M, Fig. 59, cyan) did not significantly increase the rate.
Figure 59. PDI concentration required for maximal retrotranslocation rate. RRM containing Δgpαf-BOF and 2 mM ATP were reconstituted with increasing amounts of air-oxidized PDI: total PDI used in the experiments (red, 10-20 µM); more PDI (cyan, 20-30 µM, n=2); less PDI (purple, 5 µM n=2). Each RRM was then incubated with complete cytosol at 30°C. For comparison, complete RRM incubated in the absence of all lumenal proteins are shown (green). The net αBOF-dependent spectral changes are shown after subtraction of the cytosol-free background (Fig. 39).

The optimal concentration of BiP together to determine their collective effect on Δgpαf-BOF retrotranslocation (Fig, 60; red). When these two chaperones were encapsulated together in RRM with ATP, the rate of Δgpαf-BOF retrotranslocation was essentially unchanged from that of PDI alone (Fig. 60; blue). This finding indicates that in the presence of BiP does not interfere with the PDI interaction with Δgpαf-BOF.
Figure 60. PDI and BiP combined did not increase the retrotranslocation rate compared to PDI alone. RRM containing Δgpαf-BOF and 2 mM ATP were reconstituted with both BiP (10-20 µM) and PDI (10-20 µM) (red, n=3). Each RRM was then incubated with complete cytosol at 30°C. For comparison, complete RRMs incubated in the absence of all lumenal proteins are shown (cyan), all lumenal proteins in the presence (blue) and absence of cytosol (green). The net αBOF-dependent spectral changes are shown after subtraction of the cytosol-free background (Fig. 39).

PDI binds directly to Δgpαf-BOF that lacks cystine

PDI must stimulate retrotranslocation by interacting with the ER membrane and/or the substrate. Free purified Δgpαf-BOF was therefore titrated with either reduced, oxidized PDI or untreated PDI to determine if PDI associated with ERAD substrate directly. PDI binding to Δgpαf-BOF was detected by an increase in BOF anisotropy caused by the slower rotational rate of PDI•Δgpαf-BOF than of Δgpαf-BOF alone (Fig. 61). Both oxidized and reduced PDI associated with Δgpαf (Fig. 61), but reduced PDI (Fig. 61; red) had a significantly higher affinity for this ERAD substrate than did oxidized or untreated PDI (Fig. 61; blue and black respectively) (Kd values of ~30 and
Figure 61. ∆gpαf-BOF binds more strongly to reduced PDI than to oxidized PDI. ∆gpαf-BOF (0.2 µM in Buffer A) was titrated at 4°C with oxidized PDI (0-12 µM; red; n = 6), reduced PDI (0-10 µM; blue; n = 7), or untreated PDI (0-6 µM; black; n = 7). PDI was reduced or oxidized by a 30 min, 30°C incubation in 5 mM DTT or 5 mM GSSG, respectively.

~900 nM, respectively). Thus, PDI binds tightly to ∆gpαf-BOF even though it lacks disulfides and free thiols.

In contrast, BiP did not detectably bind to ∆gpαf-BOF in the presence of ATP (Fig. 62; red). ATP-free wild type BiP (Fig. 62; blue) and a BiP mutant (T37G) (Fig. 62; orange) that has a mutation in its nucleotide binding domain and does not release its substrate showed a slightly higher affinity for ∆gpαf-BOF than did BiP with ATP (Fig. 62; red). However, ATP-free BiP still had much lower affinity for ∆gpαf-BOF than did the untreated PDI (Fig. 62; cyan). The higher affinity of these two BiPs for ∆gpαf-BOF (Fig. 62; orange and blue) is perhaps due to the inability of BiP to release its substrate in the absence of nucleotides or with T37G BiP mutation in its nucleotide-binding domain.
Figure 62. $\Delta$gpαf-BOF does not bind tightly to BiP. $\Delta$gpαf-BOF (0.2 µM in Buffer A) was titrated at 4°C with BiP and ATP (0-6 µM; red; n = 3), BiP without any nucleotide (0-6 µM; blue; n = 3), or purified BiP mutant (T37G) (0-6 µM; orange; n = 3). BiP was incubated with 2 mM ATP for 10 min, 30°C. For comparison the association between untreated, air-oxidized PDI and $\Delta$gpαf-BOF (0-6 µM; cyan; n = 7) are shown.

Does the higher affinity of $\Delta$gpαf for reduced PDI than for oxidized PDI affect the rate of retrotranslocation?

When $\Delta$gpαf-BOF was reconstituted into RRM s with ATP and either oxidized or reduced PDI, the rate of retrotranslocation was significantly lower with reduced PDI (Fig. 63; orange) than with either oxidized (Fig. 63; red) or untreated (Fig. 58; red) PDI (PDI purified using our procedures is air-oxidized). Thus, although the reduced form of PDI binds $\Delta$gpαf more tightly, maximal retrotranslocation rates require oxidized PDI and/or oxidation conditions in the lumen.
Figure 63. Maximal retrotranslocation rates require oxidized PDI. RRM containing Δgpαf-BOF and 2 mM ATP were reconstituted with: total luminal proteins (black); 10 µM oxidized PDI + 5 mM GSSG (red, n = 3); or 10 µM reduced PDI + 5 mM DTT (orange, n = 3). For comparison, complete RRMs were incubated in the absence of cytosolic proteins (green). The net αBOF-dependent spectral changes are shown after subtraction of the cytosol-free background (Fig. 39).

The association between PDI and Δgpαf-BOF was also examined by photocrosslinking and immunoprecipitation. Δgpαf-BOF was translated in vitro in the presence of [35S]Met and Nε-(5-azido-2-nitrobenzoyl)-Lys- tRNA^Lys (εANB-Lys-tRNA^Lys) that contained a photo-reactive dye (ANB). After translocation into KRM in the dark, ANB was activated by UV light to form photoadducts between radioactive any Δgpαf-BOF interacting luminal proteins. The KRM were sedimented and proteins were analyzed on SDS-PAGE gel. The photoadducts were identified by immunoprecipitation using antibodies to calnexin (αCNX), BiP (αBiP), Sec61α (αSec61), and PDI (αPDI). Antibodies to PDI were able to recognize a complex between PDI and Δgpαf-BOF (Fig. 64; red circle), which indicates that Δgpαf-BOF associates directly with PDI as was suggested by the anisotropy data (Fig. 61).
Figure 64. Direct association between Δgpαf-BOF and PDI detected by photocrosslinking and immunoprecipitation. pΔgpαf mRNA was translated in vitro in the presence of either [35S] and εANB-Lys-tRNA_Lys or Lys-tRNA_Lys as in Fig. 24 and translocated into KRMs in the dark. KRMs were sedimented and then photolyzed either before or after a 15-min 30°C incubation. Photolyzed samples were split and immunoprecipitated with affinity-purified antibodies to αSec61α, αBiP, αCNX or αPDI, as indicated. Δgpαf-PDI photoadducts are showed (●).  

RRMs do not contain any residual luminal chaperones after pH extraction  

The next question was whether XRM, after pH extraction, would still contain any residual PDI or BiP that could account for the slow release of Δgpαf-BOF from the (Fig. 54; red). RRM were reconstituted with increasing amount of purified PDI, or BiP
Figure 65. XRM do not contain any residual PDI after pH extraction. RRM were reconstituted with decreasing concentrations of PDI (lanes 1-4) or no PDI (lane 5). Then these RRM were sedimented, resuspended in SDS-PAGE buffer and run on a 10-15% SDS-PAGE gel. The amount of PDI present in these vesicles was detected by western blotting. Increasing amounts of PDI were also loaded directly the gel as control (lanes 6-8). Derlin-1, an ER membrane protein, was also detected by antibodies to Derlin-1 to show that an equivalent amount of RRM membranes was present.
Figure 66. XRMs do not contain any residual BiP after pH extraction. RRM were reconstituted with 2 mM ATP and decreasing concentrations of BiP (lanes 1-7) or no BiP (lane 8). Then these RRM were sedimented, resuspended in SDS-PAGE buffer and run on a 10-15% SDS-PAGE gel. An increasing amount of KRM (lanes 9-10) and purified BiP (lanes 11-13) were also loaded on the gel as controls. The amount of BiP present in these vesicles was detected by western blotting. Derlin-1, an ER membrane protein, was also detected by antibodies to Derlin-1 to show that each sample contained an equivalent amount of RRM membranes.

sedimented by centrifugation and run on an SDS-PAGE gel. Antibodies to either PDI (Fig. 65) or BiP (Fig. 66) were used to determine the amount of these chaperones present in the vesicles. No detectable PDI (Fig. 65; lane 5) or BiP (Fig. 66; lane 8) were in XRMs, so the only PDI or BiP that contributed to retrotranslocation of Δgpαf-BOF were added exogenously.
Discussion

The rate of Δgpαf-BOF retrotranslocation was maximal with either the full complement of lumenal proteins or with oxidized PDI. This retrotranslocation rate was greatly decreased in the absence of all soluble lumenal proteins, and by purified BiP by itself stimulated retrotranslocation only slightly over the no-lumenal-protein sample. When reduced PDI was encapsulated with Δgpαf-BOF, the retrotranslocation rate was decreased compared to that of RRMss containing oxidized PDI.

Several aspects of these data were unexpected. BiP has been shown in previous studies to be required for the retrotranslocation of several substrates in yeast (Nishikawa et al., 2005), including Δgpαf (Brodsky and McCracken, 1999; Kabani et al., 2003), yet BiP had only a small stimulatory effect on Δgpαf retrotranslocation from mammalian microsomes (Fig. 56). Also, although BiP can function as a chaperone, it appears to bind weakly, if at all, to Δgpαf (Fig. 62). The role of BiP in retrotranslocation may therefore be more complex than currently appreciated.

In contrast, only oxidized PDI was required in the lumen to obtain maximal retrotranslocation (Fig. 61). The mechanism by which PDI promotes retrotranslocation is not yet understood, but may involve PDI binding to the ERAD substrate (Gillece et al., 1999; Tsai et al., 2001). Since PDI promotes the folding of proteins that lack disulfide bonds and unreacted cystine (Cai et al., 1994; Puig and Gilbert, 1994; Wilkinson and Gilbert, 2004), it was not surprising that PDI bound Δgpαf (Fig. 61 and 64). However, the lower affinity of Δgpαf for oxidized than reduced PDI was unexpected because retrotranslocation was faster with oxidized PDI than with reduced PDI (Fig. 63). This
result suggests that tight binding of PDI to Δgpαf inhibits retrotranslocation. It is also possible that oxidized, but not reduced, PDI interacts with the retrotranslocation machinery at the membrane, and/or that the presence of 5 mM DTT in the microsomes reduces lumenal disulfide bonds and slows retrotranslocation. But since cholera toxin movement from the lumen to the cytosol was promoted by reducing agents (Tsai and Rapoport, 2002; Tsai et al., 2001; Tsai et al., 2002), PDI involvement in retrotranslocation is likely substrate-dependent.
CHAPTER VI
MEMBRANE PROTEIN REQUIREMENT FOR
THE RETROTRANSLOCATION OF A FLUORESCENCE-LABELED ERAD SUBSTRATE*

Sec61 is not essential for Δgpαf retrotranslocation

ERAD substrates must cross the ER membrane in order to degrade in the cytosol. There are several membrane proteins that were proposed to be the part of the retrotranslocation channel (Fig.16). One of the candidates that might facilitate the transport of an ERAD substrate from the lumen to the cytosol across the ER membrane is the Sec61α translocon. Other recent studies implicated Derlin-1, a membrane protein with four transmembrane segments, as a component of a retrotranslocation site (Lilley and Ploegh, 2004; Schekman, 2004; Ye et al., 2004). In order to assess the involvement of Sec61α and Derlin-1 in Δgpαf-BOF retrotranslocation, both fluorescence and photocrosslinking techniques were used.

It was previously shown that affinity-purified antibodies against Sec61α (αSec61α) blocked iodide ion passage through the aqueous pores of ribosome-free translocons (Hamman et al., 1998). It was therefore possible to use a similar approach to block the

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Figure 67. Modes of translocon pore blockage that may inhibit ∆gpαf-BOF retrotranslocation. Complete RRM s containing ∆gpαf-BOF and 2 mM ATP were reconstituted with luminal proteins and preincubated on ice with either αSec61α (A) or translating ribosome-nascent chain complexes (B). Some RRM s were also reconstituted first with the T37G BiP mutant (C) and then with luminal proteins and ∆gpαf-BOF with 2 mM ATP.

exit of an ERAD substrate from the microsomes that were preincubated with antibodies to various membrane proteins from the cytosolic side (Fig. 67). Thus, ∆gpαf-BOF retrotranslocation might be inhibited by αSec61α if retrotranslocation took place through the Sec61α translocon complex. After RRM s were preincubated with excess αSec61α at 0 °C, retrotranslocation was initiated by either the addition of EGS with a full set of cytosolic proteins (Fig. 68) or with purified 26S proteasomes (Fig. 69). The preincubation of complete RRM s with αSec61α did not alter the extent of retrotranslocation at times greater than 5 minutes rate significantly, but did show the initial rate substantially and created a transient 2-5 minute lag period (Fig. 68; right panel). This lag period was not apparent with the addition of purified 26S proteasomes (Fig. 69; right panel).
Figure 68. ∆gpαf-BOF retrotranslocation is only inhibited initially by αSec61α. On the left, complete RRMss (~10 Eq) were preincubated with 3 µg/ml αSec61α (red, n = 3) before the addition of cytosol and incubation at 30°C. For comparison, complete RRMss incubated in the presence (blue) or absence (cyan) of cytosol are shown. The longer time-scale graph is shown on the left and the shorter time-scale is on the right.

Figure 69. ∆gpαf-BOF retrotranslocation is only slightly inhibited by αSec61α in the presence of 26S proteasomes. On the left, complete RRMs (~10 Eq) were preincubated with 3 µg/ml αSec61α (red, n = 4) before the addition of purified 10 µg/ml 26S proteasomes and incubation at 30°C. For comparison, complete RRMs incubated in the presence (blue) or absence (cyan) of 10 µg/ml purified 26S proteasomes are shown. The longer time-scale graph is shown on the left and the shorter time-scale is on the right.
Figure 70. Δgpαf-BOF retrotranslocation is not blocked by RNCs. On the left, complete RRM complexes (~10 Eq) were preincubated with 10-20 nM RNCs, 40 nM SRP and 1 mM GTP (red, n = 2), or 10-20 nM non-translating ribosomes without any nascent chain and 40 nM SRP and 1 mM GTP (green, n = 2), or with only 40 nM SRP and 1 mM GTP (purple, n=2) before the addition of cytosol and incubation at 30°C. For comparison, complete RRM complexes incubated in the presence (black) or absence (blue) of cytosol are shown. On the right, the same graph is shown on an expanded scale.

Translating ribosome-nascent chain complexes (RNCs), which bind to the cytosolic side of the translocon tightly, can also block the translocon (Crowley et al., 1994; Gillece et al., 2000; Schmitz et al., 2000). Although this blockage is not stoichiometric (~50% of RRM translocons are bound under these conditions; (Hamman et al., 1998) one might expect a 50% reduction in potential retrotranslocation channels to reduce the maximal rate of retrotranslocation, if the translocons actually do constitute the sites of retrotranslocation. RNCs with an 86-residue preprolactin (pPL_{86}) nascent chain were purified by gel-filtration. pPL_{86} was translated in the presence of [^{35}S]Met to quantify RNC binding to RRM complexes by scintillation counting. This truncated version of pPL_{86} has no stop codon, so the nascent chain remains ribosome-bound at the end of the translation. These RNCs can be added back to ER vesicles in the presence of SRP and GTP to be properly targeted to Sec61α translocons. Thus, complete RRM complexes with the ERAD
substrate encapsulated inside were incubated with SRP to target the RNCs to Sec61α translocons. The resulting RRM s were incubated with complete cytosol to initiate retrotranslocation. The rate of Δgpαf retrotranslocation was not affected significantly by the tight binding of RNCs to translocons (Fig. 70; red). Ribosomes without nascent chains (Fig. 70; green), and SRP and GTP alone (Fig. 70; purple) were used as controls to exclude the possible effect of these other components.

Figure 71. Δgpαf-BOF retrotranslocation is not blocked by BiP from the lumenal side. XRMS (~10 Eq) were preincubated with 20 μM T37G BiP mutant (red, n = 5) or 20 μM wt BiP (cyan, n = 5) with 2 mM ATP before encapsulation of Δgpαf-BOF and all lumenal components to form compete RRM s. These RRM s were incubated in complete cytosol before the addition of cytosol and incubation at 30°C. For comparison, complete RRM s incubated in the presence (blue) or absence (green) of cytosol are shown. On the right, the same graph is shown on an expanded scale.

The lumenal end of the aqueous translocon pore can be closed by BiP, either directly or indirectly (Alder et al., 2005; Haigh and Johnson, 2002; Hamman et al., 1998). Whereas BiP-mediated closure is regulated and reversible under normal conditions, the pores in ribosome-free translocons are closed irreversibly if RRM s are prepared with
ATP and a T37G BiP mutant in which communication between the nucleotide and peptide binding domains is defective (Alder et al., 2005). After pH treatment, the open microsomes were incubated with T37G BiP (Fig. 71; red) or wild type BiP (Fig. 71; cyan) in the presence of ATP, and then resealed with a full set of lumenal proteins, ∆gpαf-BOF, and 2 mM ATP. When these RRM's were incubated with complete cytosol and EGS, the rate and extent of αBOF-dependent quenching did not differ significantly from samples without any extra BiP added (Fig. 71; blue). Thus, these data strongly suggest that ∆gpαf-BOF does not pass through the translocon pore during retrotranslocation, or that there is an alternative route through which retrotranslocation can take place in case of translocon blockage/occupation.

**Antibodies to Derlin-1 block ∆gpαf retrotranslocation**

In contrast, when complete RRM's were preincubated with αDer1 (affinity-purified antibodies that bind to the C-terminal region of Derlin-1), αBOF-dependent quenching, and hence ∆gpαf- BOF retrotranslocation, was reduced by more than 80% compared to positive control (Fig. 72; red). Similar results were obtained when the complete cytosol was substituted by purified 26S proteasomes (Fig. 73; red).
Figure 72. Δgpαf-BOF retrotranslocation is inhibited by αDer-1. On the left, complete RRMS (~10 Eq) were preincubated with 3- g/ml αDer1 (red, n = 6) before the addition of cytosol and incubation at 30°C. For comparison, complete RRMs incubated in the presence (blue) or absence (cyan) of all cytosolic proteins are shown. On the right, the same graph is shown on an expanded scale.

Figure 73. Δgpαf-BOF retrotranslocation is inhibited by αDer-1 in the presence of 26S proteasomes. On the left, complete RRMS (~10 Eq) were preincubated with 3 µg/ml αDer1 (red, n = 4) before the addition of 10 µg/ml purified 26S proteasomes and incubation at 30°C. For comparison, complete RRMs incubated in the presence (cyan) or absence (blue) of purified 26S proteasomes are shown. On the right, the same graph is shown on an expanded scale.
These data suggest that αDer1, but not αSec61α also blocked 26S proteasome stimulation of retrotranslocation (Figs. 68-69 and 72-73; red). Yet the αDer1 effect was more complex than simple blockage, since even after RRM were incubated with an excess of αDer1 at 0°C, the rate of αBOF-dependent quenching was initially unaltered (right panels, Figs. 72 and 73; red). But after 2 min at 30°C, Δgpαf-BOF retrotranslocation was totally blocked. Thus, αDer1 can block the retrotranslocation machinery, but only after a short lag.

**Do other derlin proteins play a role in Δgpαf-BOF retrotranslocation?**

Recently, two derlin homologues other than Derlin-1 were suggested to play a role in retrotranslocation (Derlin-2 and Derlin-3) (Oda et al., 2006). This raised the question whether the αDer would cross-react with Derlin-2 and 3 because, if so, perhaps the observed blockage of retrotranslocation cannot be attributed solely to Derlin-1 (Figs. 72 and 73). Since Derlin-2 and Derlin-3 have lower molecular weights (28 kDa and 27 kDa respectively) than Derlin-1 (29 kDa), western blotting technique was used to evaluate the cross-reaction of αDer1 with these other two proteins (Fig. 74). Since only one band was observed on the SDS-PAGE gel with the molecular weight of Derlin-1, and there were no cross-reaction between αDer1 and Derlin-2 or Derlin-3. Antibodies obtained to the C-terminal region of Derlin-2 and Derlin-3 were also tested to determine whether they could block the retrotranslocation of Δgpαf-BOF from RRM. When the RRM were preincubated αDer2, there was only a small reduction change in the retrotranslocation rate of the ERAD substrate from the ER lumen (Fig. 75; cyan) when compared to a
Figure 74. The antibodies to Derlin-1 do not cross-react with Derlin-2 and 3. Microsomal proteins were separated by SDS-PAGE, and only one band was observed after Western blotting with αDer1. Since Derlin-2 and Derlin-3 differ in molecular weight from Derlin-1, the gel shows that there is no detectable cross-reaction. According to the manufacturer of this antibody (Novus), these antibodies were raised against a peptide sequence that was present in Derlin-1, but was absent in Derlin-2 and Derlin-3.

Sample lacking αDer2 (Fig. 75; blue). Antibodies to Derlin-3 had a slightly more profound initial effect than αDer2 (Fig. 75; red) on the retrotranslocation rate, but the extent of retrotranslocation was only somewhat reduced. Thus, in contrast to αDer1, αDer2 and αDer3 have little effect on retrotranslocation. Hence retrotranslocation is blocked by antibodies specific for Derlin-1, but not for antibodies specific for Derlin-2 or Derlin-3.
Figure 75. ∆gpαf-BOF retrotranslocation is not completely inhibited by αDer-2 or αDer-3. On the left, complete RRMS (~10 Eq) were preincubated with 3 µg/ml αDer2 (cyan, n = 3), 3 µg/ml αDer3 (red, n = 3), or 3 µg/ml αDer1 (orange) before the addition of cytosol and incubation at 30°C. For comparison, complete RRMs incubated in the presence (blue) or absence (green) of all cytosolic proteins are shown.

Figure 76. ∆gpαf-BOF retrotranslocation is not completely inhibited by αHrd1. On the left, complete RRMs (~10 Eq) were preincubated with 3 µg/ml αHrd1N (N-terminal; red, n = 2), 3 µg/ml αHrd1C (C-terminal; black, n = 2), or 3 µg/ml αDer1 (cyan) before the addition of cytosol and incubation at 30°C. For comparison, complete RRMs incubated in the presence (blue) or absence (green) of all cytosolic proteins are shown.
Do antibodies to Hrd1 interfere with Δgpαf-BOF retrotranslocation?

Another important membrane complex that has been suggested to play a role in ERAD is the Hrd1 ubiquitin ligase (Bordallo et al., 1998; Kikkert et al., 2004). This protein has a short C-terminal domain in the cytosol and a large N-terminal cytosolic domain that contains the ubiquitin ligase activity. The N-terminal domain has been found to interact with several cytosolic components, including ubiquitin-conjugating enzymes and p97 (Gauss et al., 2006). Antibodies to both the C- and N-termini were obtained (αHRD1^C and αHRD1^N) and tested for their ability to interfere with the rate and extent of Δgpαf-BOF retrotranslocation (Fig. 76, red and purple). Interestingly, antibodies bound to the C-terminus of Hrd1 were slightly more inhibitory than antibodies to N-terminus (Fig. 76; black vs. red). There was an almost a complete blockage of retrotranslocation for the first 350 seconds with αHrd1^C, and both the rate and extent of retrotranslocation were less with αHrd1^C than without any antibody (Fig. 76; blue). When antibody to the N-terminus was used, a decrease in the initial rate was observed, but not to the same extent as with the C-terminus antibody. These data suggest that Hrd1 is located proximal to the site of Δgpαf. The partial inhibition prevents us from identifying a clear role for Hrd1 in retrotranslocation, in contrast to the data obtained with αDer1 that blocked retrotranslocation completely after the first few minutes.
Δgpαf photocrosslinks Derlin-1

In order to uncover which molecules were adjacent to Δgpαf during retrotranslocation, photoreactive probes were incorporated into pΔgpαf in an in vitro translation containing Nε-(5-azido-2-nitrobenzoyl)-Lys-tRNA\textsuperscript{Lys} (εANB-Lys-tRNA\textsuperscript{Lys}; (Krieg et al., 1989) (Fig. 20). After microsomes were pelleted and resuspended in complete cytosol, the sample was divided and photolyzed either before or after retrotranslocation was initiated by raising the temperature to 30°C. After the crosslinking reaction, the samples were sedimented by centrifugation and the pellet was resuspended, divided into thirds, and immunoprecipitated with antibodies to Der1, Sec61α, or TRAM, a core component of the translocon (Johnson and van Waes, 1999) (Fig. 77). A photoadduct was immunoprecipitated with αDer1 (Fig. 77; lanes 1 and 2), but not with αSec61α (Fig. 77; lanes 5 and 6) or αTRAM (Fig. 77; lanes 9 and 10). No photoadducts were seen in the absence of probe (Fig. 77; lanes 3 and 4). Since photolyzed ANB has a short reactive lifetime, photoadducts are formed only with molecules in close proximity to Δgpαf at the time of photolysis. Therefore, Δgpαf was adjacent to Derlin-1, but not detectably proximal to Sec61α or TRAM. Yet the yield of Δgpαf-Derlin-1 photoadduct was approximately the same at 0°C and 30°C (Fig. 77; lanes 1, 2), which shows that Δgpαf was adjacent to Derlin-1 both before and after retrotranslocation was initiated (see below). Since the photoreactive probes were incorporated randomly into the 9 Lys codons of Δgpαf, not all probes in a Δgpαf were adjacent to retrotranslocation site proteins at the time of photolysis. Furthermore, only a few photoreactive Δgpαf were in
Figure 77. Δgpαf photocrosslinking to Derlin-1. pΔgpαf mRNA was translated in the presence of either εANB-Lys-tRNA^{Lys} or Lys-tRNA^{Lys} as in Fig. 24 and translocated into microsomes in vitro in the dark as indicated. Microsomes were purified and then photolyzed either before or after a 15-min 30°C incubation. Photolyzed samples were split and immunoprecipitated with affinity-purified antibodies to αDer1, αSec61α, or αTRAM as indicated. Δgpαf-Derlin-1 photoadduct, •; unidentified species also seen in non-immune IgG control, ▲.

the retrotranslocation site at the time of photolysis. For these reasons, the yield of

Δgpαf-Derlin-1 photoadduct was relatively low. However, the critical observation was

that Δgpαf photoadducts were formed with Derlin-1, but not with Sec61α or TRAM.
Figure 78. Δgpαf photocrosslinking to lumenal, cytosolic, and membrane components. pΔgpαf mRNA was translated in the presence of εANB-Lys-tRNA as in Fig. 77 and translocated into microsomes in vitro in the dark. Microsomes were purified in the dark and then incubated in complete cytosol at 30°C (0' samples were on ice throughout). Sample aliquots were photolyzed at the times indicated and then split in two. One half of each aliquot was analyzed directly by SDS-PAGE (A, total sample), while the other half was sedimented to separate the microsome pellet from the supernatant prior to SDS-PAGE analysis of the radioactive species in the supernatant (B) and the microsome pellet (C). Δgpαf, ●.

The photocrosslinking samples were also examined on an SDS-PAGE gel before immunoprecipitation. Aliquots were removed at three different time-points (0’, 5’ and 15’) to examine photoadduct formation in the total sample and in the microsomes and supernatant fractions (Fig. 78). The number of Δgpαf photoadducts to cytosolic proteins increased over time, thereby indicating that the photoreactive Δgpαf was transported through the retrotranslocation site during the 30 min incubation (Fig. 78).
Is the N-terminus or C-terminus of ∆gpαf transported across the ER membrane first?

The primary ∆gpαf-BOF construct used in this project to investigate the direct involvement various components in retrotranslocation contained a BOF at the C-terminal end. But a different ∆gpαf derivative was labeled with BOF. This allowed us to determine whether the rate and extent of retrotranslocation differed for N-terminal or C-terminal probes. Since quenching of BOF only occurs upon binding to αBOF in the cytosol, comparing the rate of exposure to the cytosol and quenching by αBOF can in principle reveal which end of the ∆gpαf emerges first from the retrotranslocation site. If the N-terminally-labeled ∆gpαf is quenched more rapidly than the C-terminally-labeled ∆gpαf, then the N-terminus of the polypeptide passes solely or preferentially through the retrotranslocation site first. If there is not difference in the rate of quenching N- and C-terminal probes, then either ∆gpαf passes through the membrane in random orientation (ie. N-terminus first = C-terminus first) or in a folded conformation so that one end does not go significantly before the other. When BOF was placed on the front of the protein (T25C), and retrotranslocation was initiated by epoxomicin-treated cytosol (to avoid the fluorescence change due to substrate degradation), the initial quenching rate was about twice as fast as it was with BOF on the C-terminus (Y165C) (Fig. 79; red vs. blue). As expected, the final extent of quenching was the same for both derivatives. Thus these data strongly indicate that the N-terminal end of ∆gpαf moves preferentially through the retrotranslocation site before the C-terminal end. This result also provides experimental support for the widely-held view that retrotranslocation is an active process involving
substrate recognition and protein-mediated transport because the direction of substrate passage through the retrotranslocation site is not random. This result strongly indicates that the lumenal and/or membrane proteins that mediate substrate selection and targeting direct the N-terminus of a substrate polypeptide into the retrotranslocation site.
Discussion

The membrane proteins that form the channel through which ERAD substrates are transported from the lumen to the cytosol have not been identified. Retrotranslocation was initially proposed to occur via the Sec61α translocon pore (Pilon et al., 1998; Plemper et al., 1999a; Wiertz et al., 1996b; Zhou and Schekman, 1999), and some subsequent data are consistent with this model (Gillece et al., 2000; Kalies et al., 2005; Lee et al., 2002; Lee et al., 2004; Ng et al., 2007; Schmitz et al., 2000). Other studies have proposed that Derlin-1 and its homologues, Derlin-2 and Derlin-3, are located at the site of retrotranslocation (Lilley et al., 2006; Lilley and Ploegh, 2005; Oda et al., 2006; Ye et al., 2005). Here, we found that the rate and extent of Δgpαf-BOF retrotranslocation were not dramatically affected by closing either end of the translocon pore (Figs. 68-71). Instead, two independent techniques, Δgpαf photocrosslinking to Derlin-1 (Fig. 77) and αDer1 blockage of Δgpαf-BOF retrotranslocation (Fig. 72 and 73), strongly indicated that Derlin-1 is located at the site of retrotranslocation. These data therefore strongly suggest, but do not prove, that Derlin-1 is a structural and/or functional component of the site of Δgpαf retrotranslocation. It is also possible that more complex ERAD substrates are retrotranslocated through different retrotranslocation sites, molecular assemblies, and/or pathways.

The continuous monitoring of retrotranslocation revealed that αDer1 did not block Δgpαf retrotranslocation at \( t_0 \), but did block retrotranslocation after 2 min (Fig. 72; right). The delay presumably resulted from αDer1 being initially unable to bind to Derlin-1 or block its function when the antibodies were mixed with the RRMIs. One
possible explanation is that $\alpha$Der1 does not bind to a Derlin-1 that is already engaged with an ERAD substrate, but does bind to an unoccupied Derlin-1. Then any $\Delta$gp$\alpha$-BOF bound to the retrotranslocation site at 0°C would be transported normally to give the maximal initial rate of retrotranslocation, but further exit would be blocked when $\alpha$Der1 bound to retrotranslocation sites following substrate release. Since $\Delta$gp$\alpha$ photocrosslinking to Derlin-1 was the same at $t_0$ and at $t_{15}$ minutes (Fig. 77), $\Delta$gp$\alpha$ was positioned adjacent to Derlin-1 at both 0°C and 30°C. These combined data suggest that $\Delta$gp$\alpha$ can be trapped at an intermediate stage of retrotranslocation by varying the temperature. $\Delta$gp$\alpha$ appears to be targeted to a Derlin-1 containing retrotranslocation site at low temperature to form a membrane-bound intermediate, and only passes through the site when the temperature is raised (Figs. 72, 73, 78, 53). If so, the C-terminal end of $\Delta$gp$\alpha$ that contains the BODIPY dye must move through the retrotranslocation site in less than 2 min (Fig. 72; right). The real-time spectroscopic characterizations of retrotranslocation using various substrates and the mammalian biochemical approach described here will provide additional insights into the mechanisms that regulate and accomplish retrotranslocation.

Other membrane components that have been suggested to play a role in facilitating the transmembrane movement of an ERAD substrate have also been examined. Derlin-2 and -3 also had some effect on the initial retrotranslocation rate, but further experiments are necessary (perhaps photocrosslinking) to understand the exact nature of their involvements. Similarly, $\alpha$Hrd1 had some influence on the initial rate, but it is difficult to assess to what extent Hrd1 involved in retrotranslocation because of the intermediate
magnitude of the quenching rate. These observations perhaps indicate that Derlin-2, Derlin-3, and/or Hdr1 may be involved in either as components of the Derlin-1-containing retrotranslocation or as an alternative retrotranslocon in mammalian microsomes. The high-resolution fluorescence approach also provided some insight into how this particular ERAD substrate may leave the vesicles. By labeling either the C- or N-terminus, it was revealed that the N-terminus of $\Delta$gp$\alpha$f-BOF leaves the ER lumen first. This finding suggests that the protein is retrotranslocated as a linear polymer and that it is binding to PDI may effect this unfolding, and PDI may target the N-terminus of $\Delta$gp$\alpha$f-BOF to the membrane. Of course, other ERAD substrates might cross the membrane by the C-terminus first, and may be folded during transport. In particular, it will be very interesting to determine how glycoproteins with their large glycan moieties are able to cross the ER membrane.
CHAPTER VII

SMALL MOLECULES THAT ARE REQUIRED FOR
THE RETROTRANSLOCATION OF A FLUORESCENCE-Labeled ERAD
SUBSTRATE *

Ca\(^{2+}\) ions and their role in ER

Ca\(^{2+}\) ions have been suggested to play a role in the retention of resident ER proteins, in the export of secretory proteins, in protein folding, and in the processing and degradation of proteins in addition to the important role in cellular signaling (Sambrook, 1990). Ca\(^{2+}\) ions also play a role in Golgi protein trafficking and cargo packaging, perhaps independently from the ER. In the Golgi, Ca\(^{2+}\) ions are taken up by both SERCA and SPCAs (secretory-pathway Ca\(^{2+}\)-ATPases). Mn\(^{2+}\) ions have been also shown to influence both O and N-glycosylation in mammalian and yeast cells (Kaufman et al., 1994).

An increase in the Ca\(^{2+}\) concentration in the cytosol can be attributed in most cell types to release of Ca\(^{2+}\) from the ER. ER serves as a dynamic calcium storage facility, since the Ca\(^{2+}\) concentration inside the ER ([Ca\(^{2+}\)]\(_{ER}\)) is a 1000 fold larger than in the cytosol ([Ca\(^{2+}\)]\(_{c}\)). Changes in the Ca\(^{2+}\) levels inside or outside the organelle can control the organelle function, because [Ca\(^{2+}\)]\(_{ER}\) regulates ER homeostasis by activating the

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release or uptake channels. To date, the biophysical properties of the ER membrane are not well understood. The rate of Ca\(^{2+}\) uptake is usually controlled by \([\text{Ca}^{2+}]_{\text{ER}}\), which can range between 0.1 mM and 1mM. The mechanism by which the SERCA2b pump is regulated has been recently hypothesized (Li and Camacho, 2004) to involve the oxidoreductase Erp57 and the lumenal chaperone calreticulin. If \([\text{Ca}^{2+}]_{\text{ER}}\) is too high, it provides a driving force for Ca\(^{2+}\) movement out of the lumen. Since the release of Ca\(^{2+}\) ions from the ER is rapid and takes place through specific channels, it is likely the ER membrane has a low resting resistance or high resting conductance. This would allow the ER membrane to stabilize after rapid Ca\(^{2+}\) release. The Ca\(^{2+}\) binding proteins buffer the Ca\(^{2+}\) ions in the ER lumen (Meldolesi and Pozzan, 1998). These proteins have low affinity, but high capacity for Ca\(^{2+}\) ions. Calsequestrin and calreticulin are able to bind up to 50 Ca\(^{2+}\) ions at the same time via their acidic tails (Campbell et al., 1983). Other ER resident chaperones, including BiP, PDI, Erp72 or the membrane protein calnexin have been suggested to bind Ca\(^{2+}\) ions, although in smaller quantities than calsequestrin (Krause and Michalak, 1997; Lamb et al., 2006).

In this chapter, the involvement of Ca\(^{2+}\) ions in ERAD has been examined by reconstituting Ca\(^{2+}\) ions into the ER lumen and/or adding them to the cytosol. This approach provides the first direct evidence of the involvement and importance of a specific ionic environment in ERAD.
Ca$^{2+}$ ions in the ER lumen increase the rate and extent Δgpαf retrotranslocation

Complete RRMs were reconstituted with Δgpαf-BOF, 2 mM ATP, and concentrations of Ca$^{2+}$ ions between 0.1 mM and 10 mM to examine whether these ions have any effect on Δgpαf retrotranslocation (Fig. 80). The calcium ion concentration in

Figure 80. Δgpαf-BOF retrotranslocation rate and extent increase with increasing Ca$^{2+}$ concentration in the ER lumen. Complete RRMs containing Δgpαf-BOF and 2 mM ATP were reconstituted with the following components: no added Ca$^{2+}$ (black, n=14); 10 mM Ca$^{2+}$ (cyan, n = 9); 1 mM Ca$^{2+}$ inside (green, n = 5); 0.1 mM Ca$^{2+}$ inside (red, n = 3). Each RRM was then incubated with complete cytosol at 30°C. For comparison, complete RRMs incubated in the absence of all cytosolic proteins are shown (blue). The longer time-scale graph is shown on the left and the shorter time-scale is on the right.

the ER is thought to be 0.1-1 mM [Ca$^{2+}$]$_{ER}$ (Fig. 80; red and green), although it has also been reported to be 5-10 mM (Meldolesi and Pozzan, 1998). Thus, the range of [Ca$^{2+}$]$_{ER}$ that examined was therefore significantly above (Fig. 80; cyan) and also below (no extra Ca$^{2+}$ included, Fig. 80; black) the physiological [Ca$^{2+}$]$_{ER}$ limits. Interestingly, the 10 mM [Ca$^{2+}$]$_{ER}$ (Fig. 80; cyan) caused a small, but significant alteration in both the rate and
extent of $\Delta$gp$\alpha$f retrotranslocation from the beginning of the time-trace when compared to the control that had no Ca$^{2+}$ ions added (Fig. 80; black). Differences between 0.1 - 1 mM $[\text{Ca}^{2+}]_{\text{ER}}$ and no $[\text{Ca}^{2+}]_{\text{ER}}$ were not pronounced in the first 500 seconds (Fig. 80; red and green vs. black, right). But by 700 seconds, the RRMs with higher $[\text{Ca}^{2+}]_{\text{ER}}$ clearly exhibited a higher extent (and hence higher rate) of retrotranslocation.

**Luminal Mn$^{2+}$ ions, but not Mg$^{2+}$ ions, have the same effect as Ca$^{2+}$ ions in the ER**

To show that the rate increase seen with Ca$^{2+}$ ions was ion specific and not simply ionic-strength dependent change, RRMs were reconstituted with two different divalent cations (Mg$^{2+}$ and Mn$^{2+}$). Mn$^{2+}$ ions have a size and charge very similar to Ca$^{2+}$ ions since their size and charge are very similar, but Mg$^{2+}$ ions are much smaller (Fig. 81).

Figure 81. The rate and extent $\Delta$gp$\alpha$f-BOF retrotranslocation are increased by the addition of Mn$^{2+}$ ions to the ER lumen, but not Mg$^{2+}$ ions. Complete RRMs containing $\Delta$gp$\alpha$f-BOF and 2 mM ATP were reconstituted with the following components: 1 mM Mg$^{2+}$ (orange, $n = 3$); 1 mM Mn$^{2+}$ (red, $n = 3$); 1 mM Ca$^{2+}$ (green, $n = 3$) and no extra ions (blue, $n=14$). Each RRM was then incubated with complete cytosol at 30°C. For comparison, complete RRMs incubated in the absence of all cytosolic proteins are shown (cyan).
When RRM contained 1 mM Mg\textsuperscript{2+} ions (Fig. 81; orange), the rate of Δgpαf retrotranslocation did not differ from the observed rate with RRM that had no divalent ions included (Fig. 81; blue). In contrast, 1 mM Mn\textsuperscript{2+} (Fig. 81; red) inside the ER increased the retrotranslocation rate and extent to the same levels observed with 1 mM Ca\textsuperscript{2+} inside (Fig. 81; green). This finding suggests that Ca\textsuperscript{2+} or divalent metal ions of the same size within the ER lumen stimulate retrotranslocation.

**The absence of Ca\textsuperscript{2+} ions in the ER lumen decrease the rate of retrotranslocation**

Since neither of the RRM nor the lumen proteins were treated by chelating agents before reconstitution, it seemed likely that some endogenous Ca\textsuperscript{2+} was still present inside the RRM. However, this residual Ca\textsuperscript{2+} concentration was undoubtedly less than the physiological [Ca\textsuperscript{2+}]\textsubscript{ER} concentration. Yet because the ER lumen is a dynamic Ca\textsuperscript{2+} storage compartment, the ER chaperones and folding enzymes located in the lumen are presumably able to adapt to the fluctuation of Ca\textsuperscript{2+} concentration. Thus it was necessary to determine the retrotranslocation rate in the RRM in the absence of [Ca\textsuperscript{2+}]\textsubscript{ER} (Fig. 80). RRM were therefore reconstituted with EGTA (Ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid), a chelating agent that would preferentially bind to the Ca\textsuperscript{2+} ions on both sides of the ER membrane during the encapsulation procedure. EGTA at 5 mM would therefore bind most of any Ca\textsuperscript{2+} ions present, and would also chelate Mg\textsuperscript{2+} ions in the sample buffer. However, since the sample buffer contained 10 mM Mg\textsuperscript{2+} and 40 mM K\textsuperscript{+}, there would be sufficient Mg\textsuperscript{2+} unchelated to associate with ATP and allow it to function properly. As it is evident in Fig. 82, the initial rate of retrotranslocation with RRM that contained EGTA in the lumen (red) was significantly reduced compared to
Figure 82. The lack of Ca\textsuperscript{2+} ions in the ER lumen decreases retrotranslocation rate. Complete RRM s containing \( \Delta \text{gp}\alpha\text{f-BOF} \) and 2 mM ATP were reconstituted with 5 mM EGTA (pH. 7.5) (red, \( n = 3 \)). Each RRM was then incubated with complete cytosol at 30°C. For comparison, complete RRM s incubated in the presence (blue) or absence (cyan) of all cytosolic proteins are shown. The longer time-scale graph is shown on the left and the shorter time-scale is on the right.

that were not treated with EGTA (blue). The presence of 10 mM Mg\textsuperscript{2+} (Fig. 80; blue) instead of the 5 mM Mg\textsuperscript{2+} used previously (Fig. 39; red) did not significantly alter \( \Delta \text{gp}\alpha\text{f} \) retrotranslocation. So the observed influence of EGTA on the rate of retrotranslocation was probably due to the removal of Ca\textsuperscript{2+} ions that are somehow required to obtain maximal retrotranslocation rates.

**Ca\textsuperscript{2+} ions in the cytosol decrease the rate and extent of \( \Delta \text{gp}\alpha\text{f} \) retrotranslocation**

When \([\text{Ca}^{2+}]_c\), the Ca\textsuperscript{2+} ion concentration in the cytosol, increases dramatically due to a disturbed ER homeostasis, cell death is eventually triggered. \([\text{Ca}^{2+}]_c\) is normally 1000-fold smaller than \([\text{Ca}^{2+}]_\text{ER}\) and is maintained at this level. For example, following metabolic stimulus, the released Ca\textsuperscript{2+} ions into the cytosol from the ER are returned into the lumen via the SERCA2b pump. To determine if \([\text{Ca}^{2+}]_c\) affects \( \Delta \text{gp}\alpha\text{f} \)
Figure 83. Ca\(^{2+}\) ions in the cytosol reduce the rate and extent of Δgpαf retrotranslocation. Complete RRM were reconstituted with Δgpαf-BOF and 2 mM ATP. Each RRM was then incubated with complete cytosol at 30°C in the presence of 1 μM Ca\(^{2+}\) (green, n = 3), 0.1 mM Ca\(^{2+}\) (red, n = 3), or 0.3 mM Ca\(^{2+}\) (purple, n = 3). For comparison, complete RRMs incubated in the presence (blue) and absence (cyan) of all cytosolic proteins were incubated in the usual buffer containing 5 mM Mg\(^{2+}\). The longer time-scale graph is shown on the left and the shorter time-scale is on the right.

retrotranslocation, the cytosolic proteins were supplemented with various Ca\(^{2+}\) ion concentrations before being added to the RRMs (Fig. 83). When [Ca\(^{2+}\)]\(_c\) was present 1 μM, there was no significant change in the rate and extent of Δgpαf retrotranslocation as observed with no added Ca\(^{2+}\) (Fig. 83; green vs. blue). Thus, cytosolic Ca\(^{2+}\) at normal cytosolic conditions do not affect ERAD. However, elevating the Ca\(^{2+}\) concentration had a significant effect. When [Ca\(^{2+}\)]\(_c\) was 100-fold larger than the normal 1 μM physiological concentration, the retrotranslocation rate was slowed dramatically (Fig. 83; red). When [Ca\(^{2+}\)]\(_c\) was increased further to 300 μM (Fig. 83; purple), the amount of Δgpαf transported out of the ER lumen was further reduced when compared to cytosol containing physiological [Ca\(^{2+}\)]\(_c\) (Fig. 83; green). The increased [Ca\(^{2+}\)]\(_c\) may place the cell under stress conditions such that no further ERAD occurs in the anticipation of
imminent apoptosis. Alternatively, perhaps an ER membrane complex involved in transporting ERAD substrates through the membrane is inhibited or otherwise altered when \([Ca^{2+}]_c\) increased.

**Δgpαf retrotranslocation is maximized by removing all Ca\(^{2+}\) from cytosol**

The hemoglobin-free cytosol prepared from rabbit reticulocyte lysate is not likely to contain any \(Ca^{2+}\) ions, because the cytosolic proteins are treated briefly with 1 mM EDTA to remove any Ni\(^{2+}\) ions after their column separation from hemoglobin (see: Chapter II). This EDTA is then dialyzed out of the cytosol, so neither EDTA nor \(Ca^{2+}\) ions remain in the cytosolic protein sample. However, to determine unequivocally whether a presence of a chelating agent would alter the retrotranslocation rate, EGTA or EDTA was added to the cytosol before the initiation of ERAD (Fig. 84). Interestingly, the presence of a chelating agent in the cytosol increased the retrotranslocation rate of Δgpαf from the lumen (Fig. 84; red and green). Since placing \(Ca^{2+}\) ions on the lumenal side increased the retrotranslocation rate, in a manner very similar to placing EGTA on the cytosolic side, it is reasonable to ask whether having both lumenal \(Ca^{2+}\) ions and cytosolic EGTA in the same sample would act synergistically or independently. When 0.1 mM \(Ca^{2+}\) was enclosed in the ER lumen, and 3 mM EGTA was added to the cytosol (these experiments were done with 10 mM Mg\(^{2+}\) in both compartments), the rate of ERAD increased even further (Fig. 84; purple). Although the uncertainty of the data points shown in Fig. 84 is too large to permit an accurate quantification of the extents of retrotranslocation, the extent of \(Ca^{2+}\)\(_{in}/EGTA_{out}\) retrotranslocation is approximately the sum of the extents observed individually with \(Ca^{2+}\)\(_{in}\) and with EGTA\(_{out}\).
Figure 84. An absence of cytosolic Ca\(^{2+}\) ions the increases the rate and extent of \(\Delta\text{gp}\alpha f\) retrotranslocation. Complete RRMs encapsulated \(\Delta\text{gp}\alpha f\)-BOF and 2 mM ATP. Each RRM was then incubated with complete cytosol at 30°C with either 3 mM EDTA (green, \(n = 4\)), or with 3 mM EGTA (red, \(n = 4\)). Some complete RRMs also contained 0.1 mM Ca\(^{2+}\) inside and were incubated with complete cytosol at 30°C with 3 mM EGTA (purple, \(n = 3\)). For comparison, complete RRMs incubated in the presence (blue) or absence (cyan) of all cytosolic proteins are shown without any additional ions on either side. The longer time-scale graph is shown on the left and the shorter time-scale is on the right.

Inhibition of SERCA2b alters the rate and extent of \(\Delta\text{gp}\alpha f\) retrotranslocation

Ca\(^{2+}\) ions are transported into the ER lumen by an ATP-driven pump, SERCA2b. This pump and its functional states have been extensively investigated (Inesi et al., 2006; Ma et al., 2005). There are several inhibitors that can block the Ca\(^{2+}\) entry into the ER, thereby depleting the ER Ca\(^{2+}\) store over time. This Ca\(^{2+}\) depletion can induce a stress response, and eventually cell death. The best-known inhibitors are a sesquiterpene lactone (thapsigargin) and quinones (DBHQ).
Figure 85. Inhibition of SERCA2b alters the rate and extent of Δgpαf retrotranslocation. Complete RRM encapsulated Δgpαf-BOF and 2 mM ATP. Each RRM was preincubated with either 1 µM thapsigargin (red, n = 5), or with 1 µM butyl-hydroquinone (green, n = 3). These RRMs were then incubated with complete cytosol at 30°C. For comparison, complete RRMs incubated in the presence (black) and absence (blue) of all cytosolic proteins are shown. The longer time-scale graph is shown on the left and the shorter time-scale is on the right.

When thapsigargin or DBHQ was preincubated with complete RRMs to inhibit the SERCA2b channels, the Δgpαf retrotranslocation rate and extent both decreased (Fig. 85; red and green). This observation suggests that the SERCA2b pump has an important regulatory role in Δgpαf retrotranslocation by maintaining a high lumenal Ca$^{2+}$ ion concentration.

**Maximal Δgpαf retrotranslocation requires ATP on both sides of the ER membrane**

The ERAD substrates must cross the ER membrane in order to degrade in the cytosol. It is not known how this dislocation process is powered, although a requirement for ATP has been observed by many groups (Albring et al., 2004; Kothe et al., 2005; Lee et al., 2004; McCracken and Brodsky, 1996; Wahlman et al., 2007). To investigate
Figure 86. ATP is necessary on both sides of the ER membrane for Δgpαf retrotranslocation. Complete RRM were reconstituted with Δgpαf-BOF and either 2 mM ATP or 30 units/ml apyrase. Each RRM with 30 units/ml apyrase inside was then incubated with complete cytosol and either 30 units/ml apyrase (purple, n=7) or EGS (green, n = 5). Each RRM with 2 mM ATP inside was then incubated with complete cytosol and either 30 units/ml apyrase (red, n = 5) or EGS (black). For comparison, complete RRM incubated in the absence of all cytosolic proteins, but presence of ATP on both sides are shown (cyan). The longer time-scale graph is shown on the left and the shorter time-scale is on the right.

whether ATP is required is solely on the cytosolic or lumenal side of the membrane, apyrase was added to either or both of these compartments. When apyrase was located only in the ER lumen, the retrotranslocation rate was significantly reduced (Fig. 86; green). The rate was reduced a comparable amount when apyrase was in the cytosol (Fig. 86; red). This equivalence in reduction of retrotranslocation may indicate that ATP is required on both sides of the membrane. However, it is very possible that ATP is transported from one side of the membrane to the other during the retrotranslocation incubation.
The data revealed that ATP requirements more likely originate from the cytosolic side (less ERAD is detected) than from the lumenal side, although it is equally probable that ATP is required on both sides.

When apyrase was included on both sides to eliminate any extra ATP, which is possibly bound to many of the cytosolic, lumenal and membrane proteins, retrotranslocation was completely inhibited (Fig. 86; purple). These findings confirm the requirements for ATP during the retrotranslocation of Δgpαf.

**Discussion**

There is relatively little information about the requirement for specific ions in the ER lumen for quality control. The paucity of data can be attributed largely to the lack of available techniques that can discriminate between different ions, in the complex macromolecular system or can determine the exact ionic concentrations within the organelles. But reconstituting RRM s with different ions may reveal the importance of various ions or small molecules in the retrotranslocation of Δgpαf.

When the Ca$^{2+}$ ion concentration inside the RRM s was increased, an increase in ERAD rate was observed (Fig. 80). This finding revealed that Ca$^{2+}$ ions are involved directly or indirectly on the lumenal side to facilitate retrotranslocation. Indirect involvement might be attributable to Ca$^{2+}$ ion requirement by membrane and soluble ER lumenal proteins. The increased Ca$^{2+}$ concentration may also promote the involvement of PDI in Δgpαf unfolding and targeting (Lucero and Kaminer, 1999). It is also conceivable that the negatively charged Δgpαf binds Ca$^{2+}$ ions directly, and thereby reduce the aggregation prone nature of this ERAD substrate. The presence of Ca$^{2+}$ ions
within the lumen may also regulate the structure or functions of one or more membrane proteins, including the retrotranslocation complex.

When Ca\(^{2+}\) ions in the ER lumen were bound by EGTA, a Ca\(^{2+}\) chelator, a decrease in the export rate was observed (Fig. 82). This result could be caused by an effect of Ca\(^{2+}\) on the Δgpαf substrate, on a soluble lumenal protein such as PDI, or on a membrane component whose interaction Ca\(^{2+}\) with affects ERAD.

The Ca\(^{2+}\) effects on retrotranslocation can be duplicated by adding Mn\(^{2+}\) instead of Ca\(^{2+}\), but are not reproduced by adding extra by Mg\(^{2+}\) (Fig. 81). The observed Ca\(^{2+}\)-dependent effects are therefore divalent metal ion-specific. Since Mn\(^{2+}\) is nearly the same size as Ca\(^{2+}\), while Mg\(^{2+}\) is much smaller, Mn\(^{2+}\) and Ca\(^{2+}\) are often exchangeable in binding to proteins to elicit structural and/or functional changes (Johnson et al., 1983). However, given the large number of Ca\(^{2+}\) ion-binding proteins in the ER, it is impossible at this point to identify which proteins are increasing retrotranslocation by binding Ca\(^{2+}\).

To further explore the role of Ca\(^{2+}\) in retrotranslocation, Ca\(^{2+}\) ions were added to the cytosol (Fig. 83). When a physiological number of Ca\(^{2+}\) ions were included in the cytosol (50-100 nM), there was not any significant effect on the rate and extent of Δgpαf retrotranslocation (Fig. 82; green). However, when the cytosolic Ca\(^{2+}\) ion concentration was increased significantly upon adding much more than physiological concentration, Δgpαf ERAD rate was significantly reduced (Fig. 81; red and purple). Clearly high [Ca\(^{2+}\)]\(_{cyto}\) inhibits retrotranslocation.
Interestingly, when excess EGTA was added to the cytosol to remove any Ca\(^{2+}\) ions still present, an increase in ERAD rate was observed. It was very curious to see this rate increase, because the cytosol was already void of any Ca\(^{2+}\) ions due to a 1 mM EDTA treatment after Hb-removal and extensive dialysis. Yet, when both EGTA and EDTA were included in the cytosol in the presence of enough Mg\(^{2+}\) concentration to have functional ATP present, the decrease in retrotranslocation rate was very obvious (Fig. 83; red and green). Apparently the gradient of Ca\(^{2+}\) across the membrane is critical in determining the rate and extent of retrotranslocation. Further evidence supporting this view showed that a combined effect of 1 mM Ca\(^{2+}\) in the ER lumen and excess EGTA gave the largest Ca\(^{2+}\)-dependent change in the rate (Fig. 83; purple).

To investigate the possible involvement of the SERCA2b Ca\(^{2+}\) pump, which is an isoform of the SERCA pump family that is present in the ER membrane, several inhibitors were used. SERCA2b pump is a P-type ATPase that couples Ca\(^{2+}\) movement from the cytosol to the lumen to ATP hydrolysis. SERCA2b contains eleven (ten for SERCA2a) transmembrane helices and two large cytosolic loops (Toyoshima and Inesi, 2004; Toyoshima and Mizutani, 2004; Toyoshima et al., 2003; Toyoshima et al., 2004). SERCA2b has been shown to be upregulated by the UPR and hence may have a significant role in quality control and ERAD. The extra transmembrane segment (M11) is only present in the ER resident SERCA2b, raising the possibility that this segment may associate with resident protein of the ER membrane. One of the cytoplasmic loops binds ATP and utilizes the energy of one \(\gamma\)-phosphate hydrolysis to move two Ca\(^{2+}\) ions through the membrane. In order to accomplish this process, SERCA2a moves through
Figure 87. Putative structural changes of the Ca\textsuperscript{2+}-ATPase (SERCA2a) during its reaction cycle, based on the crystal structures in five different states. (1) Ca\textsuperscript{2+} binding to E2, which is the ground state, straightens the M5 helix and breaks the closed configuration of the three cytoplasmic domains, exposing the catalytic site. Two Ca\textsuperscript{2+} are bound in the high affinity sites formed by transmembrane helices M4, M5, M6 and M8. The cytoplasmic gate is open and bound Ca\textsuperscript{2+} exchange with those in the cytoplasm. The M1 helix is deeply embedded in the lipid bilayer, stabilized indirectly by the bound Ca\textsuperscript{2+}. (2) ATP binds and crosslinks the P- and N-domains, so that the γ-phosphate of ATP and a Mg\textsuperscript{2+} binds to the P-domain to bend it. The N-domain is fixed in a highly inclined position and makes contact with the A-domain in a strained position. The M1 helix is pulled up and bent so that the top of the transmembrane part closes the cytoplasmic gate of the Ca\textsuperscript{2+} binding sites. (3) Phosphoryl transfer to Asp 351 allows the dissociation of ADP, which triggers the opening of the N- and P-domain interface; the A-domain rotates so that the TGES loop wedges into the gap and interacts with the phosphorylation site. This causes a marked rearrangement of the transmembrane helices M1–M6; large downward movements of M4, sharp bending of M5 and rotation of M6 destroy the Ca\textsuperscript{2+}-binding sites. The lower sections of M1 and M2 push against M4L, opening the luminal gate and releasing the bound Ca\textsuperscript{2+} into the lumen. (4) The TGES loop of the A-domain fixes a particular water molecule and catalyses its attack on the aspartylphosphate. The release of the phosphate and Mg\textsuperscript{2+} unbends the P-domain. This in turn releases M1 and M2 so that M4L closes the luminal gate. The top amphipathic part of M1 (M1’) forms a part of a cytoplasmic access tunnel leading to Glu 309, the gating residue of the Ca\textsuperscript{2+} binding sites.” Figure is reprinted and text is cited from (Toyoshima et al., 2004) by the permission of Nature publishing group.
several conformational states (Fig. 87). The crystal structures of five different operational modes of SERCA2a have been determined, and it can be applied to SERCA2b. These changes mostly involve the three cytosolic domains (N, P and A), and a complicated twist in the membrane spanning helices. These various conformational intermediates are largely different from one another. The P-domain, the phosphorylation domain has very dissimilar conformations in the Ca$^{2+}$ bound and unbound states. The N-domain is the nucleotide-binding domain, while the A-domain is called the actuator domain. The N- and A-domains are connected to the rest of the proteins by flexible loops, suggesting that these domains are free to move. Among the membrane helices, six of them (M1-M6) are re-arranged upon binding to either two Ca$^{2+}$ ions or two protons (H$^+$).

Thapsigargin, a potent inhibitor of SERCA2b pump, stabilizes this protein in the Ca$^{2+}$-free conformation (Fig. 85; E2). This inhibitor dramatically reduced the rate and extent of Agpαf retrotranslocation when complete RRM s were pretreated with thapsigargin (Fig. 83; red). This finding is consistent with the requirements of a high luminal and a low cytosolic Ca$^{2+}$ result is faster retrotranslocation rate (Fig. 84; purple). Perhaps the Ca$^{2+}$-free state of the SERCA2b pump is in contact directly or indirectly with the putative retrotranslocon and inhibits it. Also, SERCA in the E2 state might be in contact with the ERAD machinery, and thereby reduce the rate. SERCA2b was found to interact with several ER resident proteins, including Erp57, CRT and CNX. These specific proteins might regulate SERCA2b function from the luminal side (Li and Camacho, 2004), since Erp57 and CRT were found to interact with SERCA2b directly
and slow down Ca\(^{2+}\) influx when \([\text{Ca}^{2+}]_{\text{ER}}\) was insufficient. These chaperones dissociate from the pump, thereby making it possible for Ca\(^{2+}\) ions to enter the lumen and replenish the Ca\(^{2+}\) store. Several other interaction partners have been also reported for SERCA2, for example Bcl2, insulin receptor substrate proteins (IRS 1 and 2) (Vangheluwe et al., 2005).

The thapsigargin results show that the relative Ca\(^{2+}\) ion concentration in the cytosol and ER lumen are somehow coupled to retrotranslocation, which cannot enter back again since the pump is inhibited. Does this mean that Ca\(^{2+}\) ions move passively from the lumen to the cytosol due to retrotranslocation and SERCA2b must actively pump Ca\(^{2+}\) ions into the lumen to maintain the \([\text{Ca}^{2+}]\) gradient? If so, thapsigargin would be expected to block retrotranslocation. Another Ca\(^{2+}\) pump inhibitor, DBHQ, had an effect similar to that of thapsigargin (Fig. 83; green), thereby confirming the importance of active Ca\(^{2+}\) pumping into the lumen for retrotranslocation. The details of Ca\(^{2+}\) involvement in retrotranslocation are still very obscure. For example, are Ca\(^{2+}\) ions important because they bind to the substrate during retrotranslocation? This seems unlikely give the spectrum of different polypeptide substrates. More likely, Ca\(^{2+}\) acts to stimulate one or more proteins or assemblies involved in retrotranslocation. Furthermore, since gradient of \([\text{Ca}^{2+}]\) across the ER membrane is required for retrotranslocation, the Ca\(^{2+}\)-sensitive protein or assembly is almost surely transmembranous. It will be very interesting to determine which component(s) requires a \([\text{Ca}^{2+}]\) gradient and why.
The dependence of retrotranslocation on cytosolic or lumenal ATP was also investigated (Fig. 84). When apyrase was encapsulated inside the lumenal without any ATP, but EGS on the cytosolic side, there was a significant reduction in retrotranslocation rate and extent even though ATP was present in the cytosol (Fig. 84; green). This decrease was much less than was seen when apyrase was present on the cytosolic side, and the RRM$s contained 2 mM ATP. This was not surprising, since it was shown earlier that the retrotranslocation of Δgpαf required the 19S RP ATPase on the cytosolic side (Lee et al., 2004; Wahlman et al., 2007) (Fig. 84; red).

The reduction of Δgpαf retrotranslocation rate was not as large when ATP was removed from either the lumen or the cytosol as it was when ATP was removed from both sides of the ER membrane (Fig. 84, purple), perhaps a small amount of ATP is transported across the membrane. This observation maybe explained if ATP is transported at a low, but significant rate across the membrane in both directions during the retrotranslocation assay. In this situation, the [ATP] maybe present in either the lumen or cytosol at a concentration that is suboptimal for extraction of this ERAD substrate (McCracken and Brodsky, 2005).

ATP in the cytosol is most likely required by the six 19S AAA proteins (Lee et al., 2004; Wahlman et al., 2007). Some ERAD substrates also employ the p97 AAA protease (Chapter I) that requires ATP for proper function (Bar-Nun, 2005; Lord et al., 2002; Rabinovich et al., 2002; Ye et al., 2001). The molecule in the lumen that requires ATP has not yet been identified for Δgpαf retrotranslocation since PDI does not bind and hydrolyze ATP. Presumably a membrane protein requires ATP during
retrotranslocation, but this has not yet been shown. BiP has been shown to be required for the retrotranslocation of many ERAD substrates (Hegde et al., 2006; Kabani et al., 2003; Plemper et al., 1997; Popescu et al., 2005) other than Δgpαf (Wahlman et al., 2007), and BiP requires ATP to function. But the steps in the retrotranslocation involve ATP have not yet been identified.
CHAPTER VIII
SUMMARY

The results of the experiments described in this dissertation have uncovered several important facts about retrotranslocation of an ERAD substrate from the ER lumen into the cytosol that would be very interesting to study further. First, the role of glycans needs more study. A mutant yeast ERAD substrate, Δgpαf, is retrotranslocated equally well from mammalian and yeast ER. Yet, the wild type protein, 3gpαf, is recognized by the mammalian quality control system as a misfolded protein and targeted back to the cytosol for degradation, but is not recognized by the yeast ERAD machinery. As a future endeavor, it would be interesting to determine whether the wild type and mutant versions take alternative routes to exit the ER.

Another ERAD substrate (A1PiZ), which is a protein with medical significance, has been shown to retrotranslocate from the ER (Kruse et al., 2006; Palmer et al., 2003). However, the reconstitution of purified A1PiZ into RRM5s will not be trivial. This protein has no disulfide bonds, but does have three glycosylation sites that complicate the purification procedure. One has to choose an overexpression system that promotes glycosylation, but does not simultaneously degrade the substrate. It is also important to control the configuration of glycans after purification, since these various forms are recognized differentially in the ER lumen, and appear to determine the ultimate fate of the protein along the secretory pathway (Cabral et al., 2001; Dejgaard et al., 2004). CPY* has been shown to be retrotranslocated to different extents when one of its four
glycans has been eliminated (Wolf and Schafer, 2005). The position of the specific glycan on CPY* also played a major role in recognition of the polypeptide by the quality control apparatus (Kostova and Wolf, 2005).

Second, the importance of cytosolic factors has been established, and they need more study. For instance, Δgpαf was not retrotranslocated in the absence of cytosol and ATP. It was later found that the sole cytosolic protein component responsible for maximal extraction of Δgpαf from the ER lumen was the 19 RP. 19S RP is an ATPase, which explained the necessity for ATP. Yet, it is possible that ATP is required on the lumenal side of the ER during retrotranslocation. It has also been shown that the retrotranslocation of many ERAD substrates requires AAA protease, p97 with or without its two cofactors (Npl4 and Ufd1) (Ye et al., 2001). Such studies are usually based on immunoprecipitation assays, and they revealed the association of 19S RP with ER membrane (Carvalho et al., 2006), but did not explain the nature of this binding. As one of the future goals, the role of 19S RP at the ER membrane can be examined to determine whether 19RP binds to one specific membrane protein in the ER membrane, as it has been proposed (Kalies et al., 2005; Ng et al., 2007), to multiple components, and/or to the membrane lipids. It is also highly likely that the 19S RP is post-translationally modified and regulated at the ER membrane (data not shown).

Third, the role of the oxidizing atmosphere in the ER lumen deserves more study since the maximal Δgpαf retrotranslocation required oxidized PDI in the ER lumen. PDI has been shown to act as a chaperone without facilitating the formation of disulfide bond formation in the ER (Cai et al., 1994; Koivunen et al., 1999; Lumb and Bulleid, 2002;
Luz and Lennarz, 1996; Noiva, 1999; Puig and Gilbert, 1994; Sun et al., 2000; Tian et al., 2004; Tsai et al., 2001; Wang and Chang, 2003; Wilson et al., 1998). Perhaps PDI is a normal part of the lumenal retrotranslocation machinery and works universally on all ERAD substrates. Examining the role of PDI in the retrotranslocation of other substrates is another area of the future research.

Fourth, the roles of Derlin-1 and other potential retrotranslocon components need to be identified. Derlin-1 was identified in yeast (Hitt and Wolf, 2004; Knop et al., 1996), and later was suggested to be the part of the retrotranslocation channel in the mammalian ER (Lilley and Ploegh, 2004; Ye et al., 2004). In this dissertation, the involvement of Derlin-1 in ∆gpαf retrotranslocation was established, although one cannot conclude from our data that this protein actually forms the channel. Identification of the protein that forms the pore in the retrotranslocon will require a combination of fluorescence and crosslinking experiments to determine both the environment (aqueous or nonaqueous) and interactions (crosslinks to the substrate or not) of various residues in Derlin-1. One can also purify and reconstitute Derlin-1 into liposomes that encapsulate fluorophores of different sizes (e.g., (Heuck et al., 2003) to determine if Derlin-1 can, by itself, form pores. But it is more the likely that the retrotranslocon has multiple membrane components (e.g. Derlin-2, Derlin-3 and/or Hrd1), and that there is more than one exit route from the ER (Mancini et al., 2003).

An alternative approach for identifying the pore-forming protein(s) is to trap an ERAD substrate during retrotranslocation by fusing it to DHFR. The binding of methotrexate (MTX) to DHFR stabilizes the formation of its globular domain and hence
blocks its passage through the membrane (Eilers et al., 1988; Schatz, 1996). Thus, an ERAD substrate and the MTX-bound DHFR domain will be halted during retrotranslocation unless the pore is huge. This chimeric ERAD substrate can therefore be reconstituted into RRM with photocrosslinking probes positioned at known sites in the Δgpαf sequence. When such a sample is illuminated with light at the end of the retrotranslocation incubation, the substrate will preferentially photocrosslink to the protein that is forming the retrotranslocon pore.

Another important and interesting finding of this study that warrants further investigation is the dependence of retrotranslocation on Ca$^{2+}$. The data discussed in Chapter VII raised the possibility that the SERCA2b pump might contribute to retrotranslocation in a very interesting way. Perhaps this protein also interacts with other lumenal, membrane and cytosolic components (Arnaudeau et al., 2002; Hojmann Larsen et al., 2001; Li and Camacho, 2004; Roderick et al., 2000; Stefanovic et al., 2004) that play a role in ERAD. Ca$^{2+}$ ions and proper homeostasis are very important for the proper functioning of ER chaperones, which might get disrupted by Ca$^{2+}$ depletion. But at present, the role(s) of Ca$^{2+}$ ions in retrotranslocation is (are) almost completely unknown.
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