Protein quality control and degradation of the Endoplasmic Reticulum: Elucidation of components and mechanisms of the retrotranslocation system

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Abbreviations

AAA	ATPase associated with a variety of cellular activities
Ab	Antibody
AB	Acrylamide Bis-acrylamide
ATP	Adenosine Triphosphate
ARS	ATP regeneration system
aa	amino acid
β-ME	*β*-mercapto-ethanol
BN	Blue Native electrophoresis
bp	base pair
CDC	"cell division cycle"
CFTR	"cystic fibrosis transmembrane conductance regulator"
CHX	Cycloheximide
CM	"complete minimal dropout"-Medium
CPY	Carboxypeptidase Y
CPY*	Mutated Carboxypeptidase Y (*prc1-1*)
DBC	Deoxy-BigCHAP
DDM	*n*-Dodecyl-β-D-maltoside
DER	Degradation in the ER
dH_2O	distilled water
DNA	Deoxyribonucleic acid
DOA	degradation of Alpha 2p
DTT	Dithiothreitol
ECL	Enhanced Chemoluminescence
EDTA	ethylenediamine-tetraacetic acid
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum associated degradation
5-FOA	5-fluoro-orotic acid
(m, µ, p) g	(milli, micro, pico) gram
GAr	Glycine-Alanine repeat
HEPES	N-(2-hydroxyethyl)piperazine-N´-(2-ethansulphate acid)
HMG-CoA	3-Hydroxy-3-methylglutaryl-Coenzyme
HRD	HMG-CoA reductase degradation
hrs	hours
HRPO	horseradish peroxidase
HTM	homologue to mannosidase I
IP	immunoprecipitation
kDa	kilo Dalton

(µ,m) l	(micro, milli) liter
LB	Luria-Bertani medium
M	Molar (1 M = 1 mol/l)
MHC	major histocompatibility complex
min	Minute
mM	millimolar (1 mM = 1 mmol/l)
MV	Minimal Medium
NaN3	Sodium azide
OD	optical density
ORF	open reading frame
P	Pellet
PBST	phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
RING	really interesting new gene
RT	room temperature
S	Supernatant
S.	Saccharomyces
SDS	Sodium dodecylsulfate
TEMED	N,N,N',N'-tetramethylethylendiamin
Tris	Tris(hydroxymethyl)amin
UBA	ubiquitin associated
UBL	ubiquitin like
UBX	ubiquitin regulatory X

Abstract

The endoplasmic reticulum (ER) is an intracellular membranous system of all eukaryotic cells into which most of the secretory proteins are imported, receive posttranslational modifications (N-glycosylation and formation of disulfide bonds), acquire their native tertiary structure and from where they are delivered to their final destination such as the ER itself, the Golgi apparatus, the vacuole, the plasma membrane, the lysosomes and the exterior of the cell. The ER contains a highly active quality control system. Malfolded proteins are selectively recognized in the ER and transported back from the ER lumen or membrane into the cytoplasm, where they are finally degraded by the 26S proteasome in an ubiquitin dependent manner. The latter process is known as ER associated degradation (ERAD). Failure of this process leads to formation of protein aggregates and results in impaired cell function. Dysfunction of ER degradation has several medically relevant implications in human cells. Therefore, it is highly important to understand the molecular mechanism of the quality control and ERAD machinery.

A number of proteins involved in the retrotranslocation process of malfolded proteins from the ER to the cytosol, such as ubiquitin conjugating enzymes Ubc1p, Ubc6p, Ubc7p and the ubiquitin protein ligases Der3p-Hrd3p or Doa10p, as well as Der1p, Kar2p and the AAA ATPase protein complex $Cdc48^{Npl4\text{-}Ufd1}$, have been identified by genetic analyses and biochemical methods. Genetic studies seem to indicate that Sec61p, the pore-forming subunit of the protein import translocon, also acts as the retrotranslocon in ER degradation as well. To understand how these components cooperatively participate in ER degradation and to get a better insight if Sec61p is really the pore-forming unit of the protein retrotranslocon, Blue Native Electrophoresis (BN) and co-immunoprecipitation techniques were applied in this study. To determine the composition of the retrotranslocon and to obtain new information on the entire process involved in ERAD, a series of CPY* fusion protein substrates with special structural characteristics were constructed and investigated in the hope of jamming the retrotranslocon and by this “catching” the retrotranslocon in the process of retrotranslocation. The strategies were as following: overexpression of CPY* in cells deleted for the ubiquitin conjugating enzymes Ubc1p and Ubc7p as well as induction of the unfolded protein response via DTT.

Using Blue Native electrophoresis, a stable Der3-Hrd3p complex was detected. The finding of the Der3-Hrd3p complex together with soluble malfolded substrates as CPY* or CPY*GFP and membrane substrates as CT* or CTG*, indicated that Der3-Hrd3p complex

acts as a part of a retrotranslocation machinery. The AAA ATPase Cdc48p was also found in this Der3-Hrd3p complex when using Blue Native electrophoresis, indicating the existence of a Cdc48-Der3-Hrd3p complex. The finding of the interaction between Cdc48 and the Der3-Hrd3p complex by co-immunoprecipitation experiments confirmed this complex composition. Blue Native electrophoresis showed two stable Sec61-Sec62-Sec63p complexes (430kDa and 480kDa), the composition of which did not undergo changes in any of the ERAD mutants, indicating that these might constitute the protein import complex. However, under overexpression conditions of CPY*-HA_3 in Δ*ubc1*Δ*ubc7* cells, defective in polyubiquitination of malfolded substrates, CPY*-HA_3 was found by Blue Native electrophoresis to be associated with these Sec61-complexes, possibly suggesting an involvement of Sec61p in the retrotranslocation process. Additionally, after DTT treatment, triggering the unfolded protein response, Der1p was also found to be associated with the Sec61-complex on Blue Native electrophoresis, showing that a dynamic recruitment of Der1p to Sec61 channel protein occurred. Further insights into the retrotranslocation process was intended to reach with the help of new ERAD substrate CPY*GAr215, a CPY* derivative containing a long Glycine-Alanine repeat sequence. The Gly-Ala repeat sequence is known to prevent degradation of the EBNA1 virus protein by the proteasome. Recently it was shown that this Gly-Ala repeat, when introduced into the proteasomal substrate ornithine decarboxylase, stalls also degradation of this enzyme, most likely due to an "idling" of the 19s cap ATPases of the proteasome unable to "grab" the unstructured, "slippery" Gly-Ala repeat. Previous work by Thomas Bohnacker (Diplomarbeit, Universität Stuttgart, 2004) has indicated that full length CPY*GAr215-HA_3 is only "processed". A partial degradation product appeared, displaying a loss of the C-terminal part of the protein. Full length and the intermediates of CPY*GAr215-HA_3 were shown here to be glycosylated and located in ER vesicles. It was observed here that there is a slight growth reduction in temperature sensitive *Sec61-2* cells overexpressing CPY*GAr215 at 35°C. Sec61p was found to interact with Cdc48p and both, full length and intermediates of CPY*GAr215 by co-immunoprecipitation experiments. Remarkably, Blue Native electrophoresis also indicated the existence of a Sec61-Cdc48-CPY*GAr215 complex in wild type cells. All together, this work suggests the existence of a Sec61-Cdc48-Der3/Hrd3-CPY*GAr215 complex which might form a dynamic retrotranslocon in ER degradation.

With the help of biochemical methods, the results in this study show the existence and dynamic changes of Cdc48-Der3-Hrd3-substrate protein complexes. This might constitute at least in part the scaffold of the retrotranslocon. Sec61p, as a pore-forming protein, might be

involved in the retrotranslocation process. Cdc48 protein complex might act as a linker between the pore and ubiquitination system, and the proteasome, handing over the ubiquitinated substrates from cytosolic face of the ER to 26S proteasome for final degradation.

Zusammenfassung

Das endoplasmatische Retikulum (ER) ist ein intrazelluläres Membransystem in eukaryontischen Zellen, in das der Großteil der sekretorischen Proteine importiert wird. Diese Proteine erhalten dort postranslationale Modifikationen (N-glycosylierung und Disulfidbrückenbildung) und ihre natürliche tertiäre Struktur, bevor sie zu ihren endgültigen Zielen wie z. B. das ER selbst, dem Golgi Apparat, der Vakuole, der Plasmamembran, dem Lysosom oder in den extrazellulären Raum exportiert werden. Das ER hat ein sehr aktives Qualitätskontrollsystem. Falschgefaltete Proteine werden selektiv im ER erkannt und vom ER Lumen oder der ER Membran zurück ins Zytoplasma transportiert, wo sie dann schließlich vom 26S Proteasom in einem ubiquitinabhängigem Prozess abgebaut werden. Dieser Prozess ist bekannt als ER assoziierte Degradation (ERAD). Fehler in diesem Prozess führen zu Proteinagregation, was schließlich zu einer Beeinträchtigung der Zellfunktionen führt. Eine Fehlfunktion der ER assoziierten Degradation hat mehrere medizinisch wichtige Auswirkungen in menschlichen Zellen. Daher ist es sehr wichtig die molekularen Mechanismen der Qualitätskontrolle der ERAD Maschinerie zu verstehen.

Eine Anzahl an Proteinen die an dem Retrotranslokationsprozess von falschgefalteten Proteinen vom ER zum Zytoplasma beteiligt sind, so wie z.B. Ubc1p, Ubc6p, Ubc7p und die Ubiquitin Protein Ligasen Der3p-Hrd3p oder Doa10p, als auch Der1p, Kar2p und der AAA ATPase Protein Komplex $Cdc48^{Np14\text{-}Ufd1}$ wurden durch genetische und biochemische Analysen identifiziert. Genetische Studien scheinen darauf hinzuweisen, dass Sec61p, die porenbildende Untereinheit des Proteinimporttranslocons, auch als Retrotranslocon bei der ER Degradation dient. Um zu verstehen, wie diese Komponenten miteinander bei der ER Degradation mitwirken, und um einen besseren Einblick zu bekommen, ob Sec61p wirklich eine porenbildende Einheit des Proteinretrotranslokons ist, wurden Blue Native Elektrophorese (BN) und Coimmunoprezipitationstechniken in dieser Studie angewendet. Um die Zusammensetzung des Retrotranslocons zu bestimmen, als auch um neue Informationen über den gesamten Prozess der im ERAD abläuft zu erhalten, wurden eine Reihe von CPY* Fusionsproteinsubstraten mit speziellen strukturellen Charakteristika konstruiert. Diese wurden in der Hoffnung das Retrotranslokon zu blockieren untersucht, um so das Retrotranslokon im Moment der Retrotranslokation zu „erwischen". Die Strategie war die Folgende: Überexpression von CPY* in Zellen, in denen die Ubiquitin konjugierenden Enzyme Ubc1p und Ubc7p deletiert sind, als auch die Induktion der „Unfolded protein response" durch DTT.

Durch den Einsatz von Blue Native Elektrophorese wurde ein stabiler Der3-Hrd3p Komplex detektiert. Die Entdeckung des Der3-Hrd3p Komplexes zusammen mit löslichen falschgefalteten Substraten wie CPY* oder CPY*GFP und Membransubstrate wie CT* oder CTG*, deuten darauf hin dass der Der3-Hrd3p Komplex als ein Teil der Retrotranslokationsmaschinerie dient. Die AAA ATPase Cdc48p wurde in diesem Der3-Hrd3p Komplex durch Blue Native Elektrophorese gefunden, was auf die Existenz eines CDC48-Der3-Hrd3p Komplexes hindeutet. Die Entdeckung einer Interaktion zwischen Cdc48 und des Der3-Hrd3p Komplexes durch Coimmunoprezipitationsexperimenten bestätigte diese Komplex Zusammensetzung. Blue Native Elektrophorese zeigte zwei stabile Sec61-Sec62-Sec63p Komplexe (430kDa und 480kDa), eine Zusammensetzung die keiner Veränderung in irgendeiner der ERAD Mutanten unterlag, was darauf hindeutet dass diese den Proteinimportkomplex bilden. Unter Überexpressionsbedingungen von CPY*-HA_3 in *Δubc1Δubc7* Zellen, welche einen Defekt bei der Polyubiquitinierung von falschgefalteten Proteinen haben, zeigte sich allerdings, dass CPY*HA_3 mit diesen Sec61-Komplexen assoziiert sind, was durch Blue Native Elektrophorese gezeigt wurde. Das deutet auf eine Beteiligung von Sec61 am Retrotranslokationsprozess hin. Außerdem stellte sich nach DTT Behandlung zur Auslösung der „Unfolded protein response" durch Blue Native Elektrophorese heraus, dass Der1p auch mit dem Sec61 Komplex assoziert ist. Dies zeigt, dass eine dynamische Rekrutierung von Der1p zum Sec61 Kanalprotein auftrat. Weitere Einblicke in den Retrotranslokationsprozess wollte man mittels des neuen ERAD Substrates CPY*GAr215, einem CPY* Derivat mit einer langen Glycine-Alanine Wiederholungssequenz, erreichen. Die Gly-Ala Wiederholungssequenz ist dafür bekannt, dass sie den Abbau des EBNA1 Virusproteins durch das Proteasom verhindert. Vor kurzem wurde gezeigt, dass diese Gly-Ala Wiederholungen, wenn sie an das Proteasom Substrat Ornithine Decarboxylase gehängt werden, den Abbau auch von diesem Enzym verhindert, was wahrscheinlich dadurch verursacht wird, dass durch ein „Leerlaufen" der 19S Cap ATPasen des Proteasoms es unmöglich wird, die unstrukturierten „rutschigen" Gly-Ala Wiederholungen zu „packen". Dei frühere Arbeit von Thomas Bohnacker (Diplomarbeit, Universität Stuttgart, 2004) zeigte, dass das gesamte CPY*GAr215-HA_3 nur „prozessiert" wird. Ein Zwischenabbauprodukt tauchte auf, was einen Verlust des C-terminalen Teiles des Proteins anzeigt. Das gesamte Protein und auch die Intermediate vom CPY*GAr215-HA_3 stellten sich als glycosiliert und in ER Vesikeln lokalisiert heraus. Es zeigte sich durch Coimmunoprezipitationen, dass Sec61p mit Cdc48p interagiert, sowie auch mit dem gesamten Protein und auch den Intermediaten von CPY*GAr215-HA_3.

Bemerkenswerterweise deutete sich auch durch Blue Native Elektrophorese die Existenz eines Sec61-Cdc48-CPY*GAr215 Komplexes in Wildtypzellen an. Zusammengefasst deutet diese Arbeit auf Existenz eines Sec61-Cdc48-Der3/Hrd3-CPY*GAr215 Komplexes hin, welcher wohl einen dynamisches Retrotranslokon für die ER Degradation bilden kann.

Mit der Hilfe von biochemischen Methoden, zeigen die Ergebnisse dieser Studie die Existenz und die dynamischen Veränderungen des Cdc48-Der3-Hrd3-Substrat Protein Komplexes. Dies könnte zu mindest in Teilen das Gerüst des Retrotranslokons darstellen. Sec61p, konnte als ein Porenbildendes Proteien an dem Retrotranslokationsprozess beteiligt sein. Der Cdc48 Proteinkomplex könnte als ein Linker zwischen der Pore, dem Ubiquitinierungssystem und dem Proteasom fungieren, indem es die ubiquitinierten Substrate von der zytosolischen Seite des ERs zum 26S Proteasom zur endgültigen Degradation überreicht.

1. Introduction

1.1 ER quality control

1.1.1 Import of nascent proteins into the ER

The endoplasmic reticulum (ER) is a membranous system present in all eukaryotic cells, necessary for the delivery of secretory proteins to their final destination such as the ER itself, the Golgi apparatus, the plasma membrane, the lysosomes and the exterior of the cell (Rapoport et al., 1996). To enter the secretory pathway, nascent proteins are translocated into the ER from the cytosol either co- or post-translationally. Both co- and post-translational passage of polypeptide chains across the ER membrane are mediated through a hydrophilic translocation channel. The channel not only translocates soluble secretory proteins but also integrates membrane proteins into the lipid bilayer. Sec61p is an essential polytopic membrane protein with ten transmembrane domains which creates the protein-conducting channel in the ER membrane. For both translocation routes, the Sec61 heterotrimeric complex, in yeast consisting of the proteins Sec61p, Sbh1p, and Sss1p, which correspond to Sec61α, Sec61β and Sec61γ respectively in mammals, constitutes the membrane channel. The Sec61 channel, being an oligomer formed from three or four copies of a Sec61p-Sbhp-Sss1p (Sec61α-Sec61β-Sec61γ) heterotrimer, mediates cotranslational protein import into the ER in both yeast and mammalian cells (Corsi and Schekman 1996; Rapoport et al., 1996; Beckmann et al., 2001; Morgan et al., 2000). In yeast, the Sec61 complex can associate with the Sec63 complex, consisting of the ER membrane proteins Sec62p, Sec63p and Sec71p, together with the peripheral membrane protein Sec72p, to form the heptameric Sec complex responsible for post-translational protein import into the ER (Johnson and van Waes, 1999). In addition, there is a second heterotrimeric ER membrane located protein complex. A Sss1p-containing complex together with a Sec61p homologue, Ssh1p, and a Sbh1p homologue, Sbh2p in yeast, named as Sss1p-Ssh1p-Sbh2p complex, is involved in a co-translational transport pathway and might be functionally redundant with the Sec61p complex (Finke et al 1996). The ER luminal chaperone Kar2p/Bip binds to the luminal, DnaJ-like domain of Sec63p in an ATP dependent manner and aids the translocation process. Soluble secretory polypeptides are delivered directly into the lumen of the ER. Integral membrane proteins, both single- and multispanning, use the translocation channel for their insertion into the lipid bilayer as well. Multiple structural and functional states of the translocon have been detected, which revealed that the structure of the translocon is highly flexible and very dynamic. Its structure and,

hence, function is strongly regulated (Römisch, 1999; Johnson and Haigh, 2000; Beckmann et al., 2001).

1.1.2 The ERAD machinery

Proteins enter the ER in an unfolded state and are subsequently folded within this organelle by a highly efficient folding machinery. Failure to fold properly leads to impaired cell function. It is, therefore, imperative that the ER contains a system able to discriminate between properly folded and modified, and terminally misfolded proteins. This discrimination must be initiated by the discovery and retention of the malfolded proteins in the ER, which is then followed by their degradation. At the same time, the ER must be able to regulate the function of its resident proteins upon incoming internal and external signals. The available data suggest that proteins have to undergo a complete cycle of progressive maturation and quality control at each individual step, before reaching a 'checkpoint' at which the decision is made about their ultimate fate: packaging into ER-to-Golgi transport vesicles or retrograde transport to the cytosol for degradation. The various recognition and targeting events as a whole have been referred to as the 'quality control' function of the ER (Hammond and Helenius, 1995; Ellgaard and Helenius, 2003). The findings, in *S. cerevisiae*, that the misfolded vacuolar peptidase CPY* (Hiller *et al*., 1996) and the mutant secretory protein pro-α-factor (Werner *et al*., 1996) were retained in the ER lumen and degraded in the cytoplasm by the proteasome, were crucial in establishing the concept of retrograde transport of malfolded proteins out of the ER. Recent studies have revealed that a process called ER-associated degradation (ERAD) or simply ER degradation, by which malfolded proteins are degraded by the proteasome or the ubiquitin-proteasome system in the cytosol, is responsible for the elimination of malfolded proteins as well as of regulated enzymes of the ER membrane (Sommer and Wolf, 1997; Plemper and Wolf, 1999; Brodsky and McCracken, 1999) (Fig.1).

1.1.2.1 Retrotranslocation from the ER to the cytosol

Genetic studies in yeast revealed a significantly retarded degradation of CPY* (Plemper et al., 1997), mutated pro-α-factor (Pilon et al., 1997) and a mutant polytopic membrane protein, Pdr5* (Plemper et al., 1998), in mutants conditionally defective in the Sec61 translocon. Co-immunoprecipitation studies indicated that Sec61β is associated with the major histocompatibility complex class I (MHC class I) heavy chain upon the action of cytomegalovirus (Wiertz et al., 1996a). Sec61β was furthermore found to be associated with wild-type and mutant forms of CFTR (Bebök et al., 1998) and with a truncated variant of the rough ER-specific type I transmembrane glycoprotein ribophorin I (RI332) (de Virgilio et al.,

1998) during their dislocation from the ER. These results suggested that the Sec61 translocon may constitute also the retrograde transport channel for malfolded soluble and membrane proteins back into the cytoplasm for degradation or is part of such a channel (Pilon et al., 1997; Plemper et al., 1997; Zhou and Schekman, 1999). However, it is very likely that the retrotranslocation channel differs in its composition from the protein import channel. Deletion of *SBH1*, *SBH2* or *SSH1* had no effect on export of a soluble misfolded form of CPY* from the ER (Plemper et al., 1997). Analysis of a temperature sensitive allele of *SEC62* defective in protein import into the ER suggests that Sec62p is not required for export as well (Pilon et al., 1997; Plemper et al., 1997). Deletion of *SEC71* leads to an unstable Sec72p (Feldheim and Scheckman 1994) and this protein was also shown to be dispensable for export (Plemper et al., 1997). Due to the involvement of Kar2p which is anchored to the Sec complex via Sec63p, in the degradation of soluble but not membrane anchored proteins, it is still controversial if the requirement of Sec63p for export is necessary or not (Plemper et al., 1997). The large pore size (20-40 Å) of the Sec61 channel (Hanein et al., 1996; Hamman et al., 1997) could allow the dislocation of even fully folded and glycosylated protein domains across the ER membrane. It was demonstrated that malfolded glycosylated proteins destined for degradation via the ubiquitin-proteasome system leave the ER in a glycosylated form (Hiller et al., 1996; Wiertz et al., 1996b). Experiments indicate that these ERAD target glycoproteins are deglycosylated in the cytoplasm prior to the degradation by the proteasome (Suzuki et al., 2000). A recent report suggests that a fusion protein of enhanced GFP (EGFP) with the MHC class I heavy chain (EGFP-HCI) domain may be retrotranslocated without unfolding of the EGFP domain (Fiebiger et al., 2002). The inner diameter of the translocon pore is estimated to fluctuate between 15Å, in its inactive state, and 40-60 Å during protein translocation (Johnson and van Waes, 1999; Haigh and Johnson, 2002). Therefore, it is possible that in its active state the translocon could be large enough to allow the passage of molecules that correspond in size to proteins which are still partially folded or glycoproteins with their bulky sugar moieties attached. Nevertheless, the retrotranslocon may even accommodate larger molecules through rearrangements of translocon subunits. In this case, however, accessory factors should be involved in regulating specificity and directionality of this Sec61 pore and thus allow protein import and export via differently assembled channels. Recently, Derlin-1, the yeast Der1p (Knop et al., 1996) homolog in mammals, was found in association with VCP/p97, US11 and with both glycosylated and deglycosylated forms of the MHC class I molecules. Due to the presence of four transmembrane domains in Der1p, it was proposed that Derlin-1 may constitute part of a retrotranslocon for dislocation of a special subset of ER proteins in

mammals (Ye et al., 2004; Lilley and Ploegh, 2004). However, no retrotranslocated protein has been trapped in this channel yet. Therefore, further experiments have to prove this hypothesis. So far, all data indicate a great plasticity of the retrotranslocon for ERAD. Therefore, additional convincing biochemical proof is essential for the characterization of the dislocation channel.

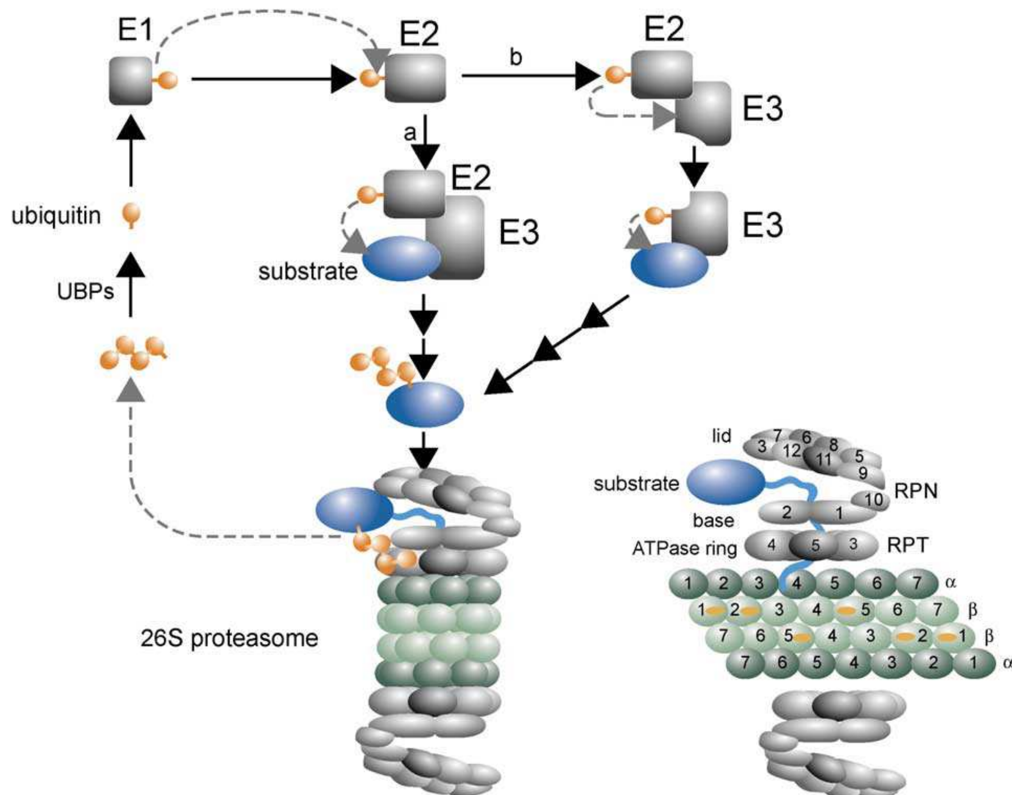


Figure 1. The pathway of ubiquitin-linked degradation of proteins and the 26S proteasome. After linkage of a polyubiquitin chain onto the protein to be degraded, its recognition occurs at the 19S cap of the 26S proteasome, which is composed of 12 Rpn (non-ATPase) and 6 Rpt (ATPase) subunits. Degradation is brought about by the proteolytic activity of a pair of three different β-subunits each located in the cylinder of the 20S $\alpha7\beta7\beta7\alpha7$ core unit of the 26S proteasome (inset after Wolf and Hilt, 2004)

Following retrotranslocation from the ER, nearly all the misfolded polypeptides or unassembled secretory soluble and transmembrane proteins are polyubiquitinated and finally degraded by the ubiquitin-proteasome system (UPS) in the cytoplasm (Biederer et al., 1996; Hampton et al., 1996; Hiller et al., 1996; Plemper et al., 1998; Werner et al., 1996). Ubiquitin triggered protein degradation by the proteasome takes place after ubiquitin activation, ubiquitin conjugation and ligation of ubiquitin onto the substrate, which are carried out by the

ubiquitin activating enzyme (Uba/E1), ubiquitin conjugating enzymes (Ubc´s/E2´s) and ubiquitin-protein ligases (E3´s), separately (Fig. 1). The substrates to be degraded have to be marked with a chain of four or more ubiquitin moieties by formation of an isopeptide-bond between the carboxyl-terminal glycine of ubiquitin and an ε-amino group of an internal lysine residue of the substrate or its amino terminus (Wolf and Hilt, 2004).

In the recent years, genetic screening of various yeast mutants had identified a number of protein components involved in ERAD (Fig. 2). Ubc1p, Ubc6p and Ubc7p were found to be ubiquitin-conjugating enzymes, which act on ERAD substrates in the yeast *Saccharomyces cerevisiae* (Hiller et al., 1996; Biederer et al., 1996; Friedländer et al., 2000; Gardner et al., 2000). Ubc6p is tail-anchored in the ER membrane with its active site facing to the cytosol. Ubc1p and Ubc7p are soluble enzymes. While there is no known membrane partner for Ubc1p, Ubc7p activity requires the presence of the membrane anchor Cue1p, which recruits the cytoplasmic enzyme to the ER membrane (Biederer et al., 1997). The RING-H2-finger protein Der3p/Hrd1p was identified as a polytopic, ER-membrane located ubiquitin-protein ligase (E3), which functions together with the E2´s Ubc7p or Ubc1p in polyubiquitinating the retrotranslocated substrates (Bays et al., 2001; Deak and Wolf, 2001). Another ER membrane spanning protein, Hrd3p, which interacts with Der3/Hrd1p, is also necessary for the ER degradation of many proteins (Deak and Wolf, 2001; Gardner et al., 2000; Plemper et al., 1999). Studies on the retrotranslocon have identified a genetic interaction between Hrd3p, Der3p and Sec61p respectively (Plemper et al., 1999a). It was recently shown that Ubc6p undergoes rapid turnover and that its degradation is independent of the Sec61 translocation channel. Although Ubc6p requires Ubc7p for proteasomal degradation, its elimination is independent of Der3/Hrd1p (Walter et al., 2001). Instead, it requires a new RING-H2 containing E3 of the ER membrane, identified as Doa10p (Swanson et al., 2001). Loss of either Der3p or Doa10p causes only a modest increase of unfolded proteins in the ER, which, in turn, leads to a mild activation of the unfolded protein response, while loss of both proteins at the same time exhibits a severe increase in malfolded proteins (Swanson et al., 2001). The mannosidase-like protein Mnl1p/Htm1p has recently been described to function as a lectin and being involved in the degradation of glycoproteins such as CPY* (Jakob et al., 2001; Nakatsukasa et al., 2001). Another important finding has been the identification of the Cdc48p/Ufd1p/Npl4p complex, crucial for “pulling” of malfolded proteins out of the ER lumen or away from the ER membrane (Ye et al., 2001; Hampton et al., 2001; Jarosch et al., 2002; Rabinovich et al., 2002; Richly et al., 2005). Interestingly, several differences have been found between soluble and membrane proteins regarding their requirements for ERAD

components. For example, while the elimination of soluble, ER-luminal CPY* required an additional ER-membrane protein, Der1p (Knop et al., 1996), the ER/Golgi calcium pump Pmr1p (Dürr et al., 1998), and the major Hsp70 chaperone of the ER, Kar2p (Plemper et al., 1997), degradation of the polytopic membrane protein Pdr5* as well as HMG-R2, did not depend on the presence of these particular proteins (Gardner et al., 2000; Plemper et al., 1998). Given the cooperation between targeting, dislocation and degradation of malfolded proteins, it is very likely that these components mentioned above do not act independently of each other.

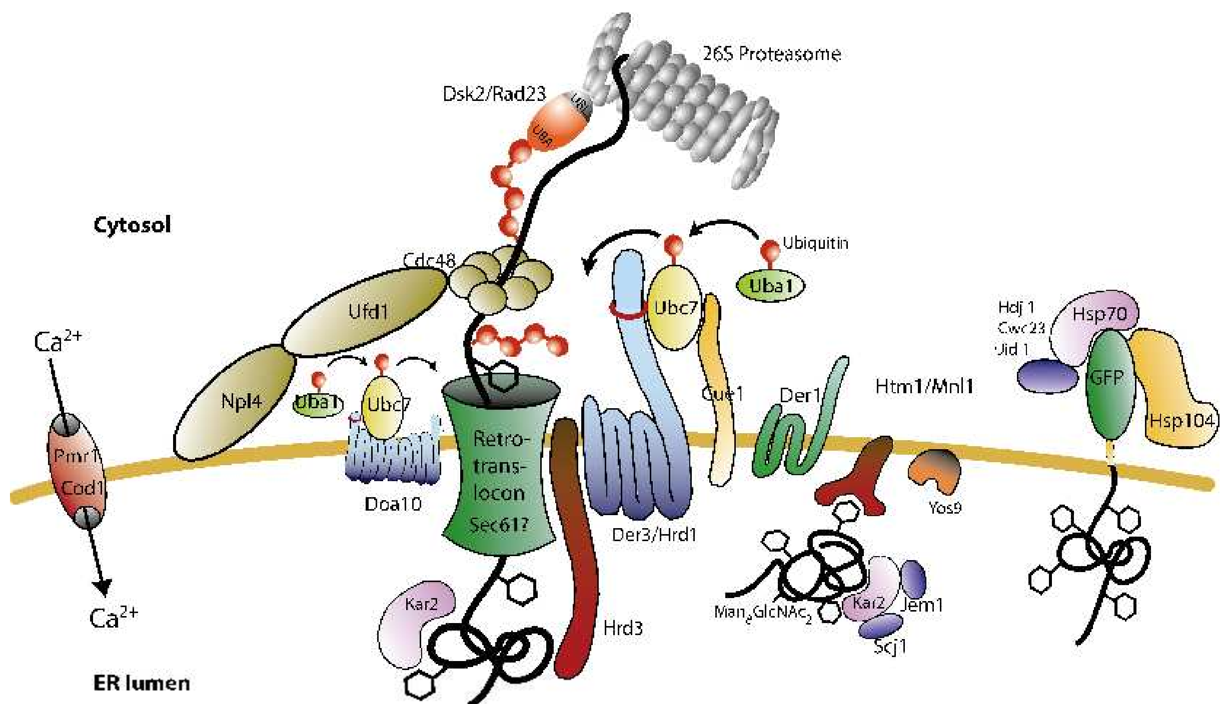


Figure 2. ER-associated protein degradation machinery. See text (modified after Kostova and Wolf, 2003)

1.1.2.2 Function of AAA ATPase Cdc48 in ER associated degradation

Once malfolded or orphan proteins of the ER are recognized by the ERAD machinery and targeted to the retrotranslocon, their translocation into the cytoplasm requires a pulling (cytoplasm) or pushing force (ER lumen) to direct the substrate to the proteasome. A defect in polyuniquitination not only arrests the degradation of substrates via the proteasome (Hiller et al., 1996; Biederer et al., 1997) but also fails to extract substrate out of the ER membrane to the cytoplasm. The polypeptide segment seems to become stuck in the ER membrane or slide back into the ER lumen (Biederer et al., 1997; Bordallo et al., 1998; Shamu et al., 2001). Inhibition of proteolytic activity of proteasomes leads to the accumulation of non-degraded

substrates, such as US11-mediated turnover of MHC class I heavy chain, in the cytosol, rather than in the ER (Wiertz et al., 1996; Ward et al., 1995; Huppa et al., 1997; Yang et al., 1998; Kikkert et al., 2001). Also, mutation of the 19S regulatory particle doesn't prevent the retrotranslocation of yeast CPY* from the ER to the cytosol (Jarosch et al., 2002). This data strongly suggests that it is the progressive polyubiquitination rather than the proteasome itself which might play an important role in a kind of ratcheting mechanism moving the substrate from the retrotranslocation channel into the cytoplasm. The finding that a hypo-ubiquitinated CPY* fails to be completely transported into the cytosol (Jarosch et al., 2002) is consistent with this proposal. Recently much attention has been given to the highly conserved AAA ATPase — Cdc48 in yeast or the valosin-containing protein (VCP)/p97 in mammals. Cdc48/p97, like most AAA proteins, forms homo-hexamers comprising an N-domain and two AAA domains, D1 and D2 (Zhang et al., 2000; Huyton et al., 2003; DeLaBarre and Brunger, 2003; Davies et al., 2005). While AAA domains undergo strong conformational changes upon ATP hydrolysis (Zhang et al., 2000; Rouiller et al., 2000), the N-domain interacts with a number of adaptor proteins that are believed to direct the chaperone activity of the proteins into distinct pathways. p97 is known to function together with the cofactor p47, which interacts with ubiquitin via its ubiquitin associated domain (UBA), in the reconstitution of the ER and Golgi apparatus following cell division (Kondo et al., 1997; Rabouille et al., 1998). p47 and its yeast homologue Shp1p possess an " ubiquitin regulatory X (UBX) " domain. Like UBL domains, UBX domains are closely related to ubiquitin in structure (Buchberger et al., 2001; Dreveny et al., 2004), although they show scarce sequence-relatedness to ubiquitin. Recent studies also show that Cdc48p interacts with Shp1/p47 via its UBX domain. The p97/p47 complex has been shown to bind ubiquitinated proteins in vivo, and to mediate the degradation of both model and native substrates in the fusion of Golgi membranes (Hartmann-Petersen et al., 2004; Schuberth., et al; 2004). Several lines of evidence now indicate that Cdc48/p97p, in both yeast and mammals, functions together with two other cofactors, Npl4p and Ufd1p, which contain neither UBX nor UBA domains, mediating the cytosolic extraction and degradation of a number of misfolded ER-proteins in an ATP-dependent manner and deliver them to the downstream receptors Rad23p and Dsk2p, which hand the substrates over to the proteasome (Bays et al.,2001a; Ye et al., 2001; Braun et al., 2001; Jarosch et al., 2002; Rabinovich et al., 2002; Gnann et al., 2004; Medicherla et al., 2004; Richly et al., 2005). This Ufd1-Npl4 protein complex binds ubiquitinated proteins through a UT3 domain in Ufd1p and an NZF domain in Np14p, although budding yeast lacks the canonical NZF domain (Meyer et al., 2002; Ye et al., 2003; Alam et al., 2004). To direct the substrates to the proteasome,

Cdc48/p97 might interact with polyubiquitin chains of polypeptides via its ND1 domain and the "cofactors" Ufd1p-Npl4p. Interaction of ubiquitin with the UT3 domain of Ufd1p enhances Cdc48/p97-polyubiquitin binding. The findings that a new RING-H2 containing E3 enzyme gp78 physiologically and functionally interacts with p97 (Zhong et al., 2004), and, that a newly identified ER membrane protein, VIMP, appears to recruit VCP/p97 to Derlin-1 in the ER membrane (Ye et al., 2004), suggest that the Cdc48p$^{Ufd1\text{-}Npl4}$ complex may serve a dual role at the ER: it may interact with non-ubiquitinated substrates very early during dislocation, contribute to their extraction from the membrane and facilitate their ubiquitination by ER-resident E3 enzymes. Subsequently, it may bind to the polyubiquitinated substrates with higher affinity, release them from the membrane and keep them in a degradation-competent conformation. The polyubiquitylated substrates are then handed over to the 26S Proteasome via the UBA-UBL domain containing proteins Rad23 and Disk2 (Medicherla et al., 2004; Richly et al., 2005), where they are completely unfolded and finally degraded into fragments of between 3 and 25 amino acids in size. Recently, in yeast the ER membrane protein Ubx2p was found to confer interaction of the Cdc48 complex and the ubiquitin ligases Der3p/Hrd3p and Doa10p (Neuber et al., 2005; Schuberth and Buchberger, 2005).

1.1.2.3 The proteasome

With a total molecular mass of approximately 1.2 MDa, the eukaryotic 26S proteasome consists of two parts, the 20S core and the 19S regulatory particle (Fig. 1). The 20S core is a cylinder composed of four stacked rings, each containing seven different α subunits or seven different β subunits, with an overall $\alpha 7\beta 7\beta 7\alpha 7$ geometry. Three different active sites, a chymotrypsin-like, a trypsin-like and an acidic (PGPH) site, are located within a pair of three different β rings. β1/Pre2p, β2/Pup1p and β5/Pre3p constitute the subunits containing active sites of the proteasome (Heinemeyer et al., 1997; Arendt and Hochstrasser, 1997). Mutations in the subunit of the chymotrypsin-like activity (Pre2) cause the most severe inhibition of proteolytic activity of the proteasome. A mutation in a neighbouring subunit Pre1, also causes a marked decrease in the rate of degradation of ER-target proteins due to disturbance of the chymotrypsin-like activity sites within subunit Pre2p (Biederer et al., 1996; Hiller et al., 1996; Werner et al., 1996; Arendt and Hochstrasser, 1997). The 19S cap, functionally divided into two parts, the base and the lid, is involved in the recognition, binding and unfolding of ubiquitinated proteins, as well as in the regulation of the opening of the gate of the 20S core particle. Mutations in two ATPase subunits of the yeast 19S particle Cim3p (Rpt6) and Cim5p (Rpt1) also reduce the rate of ERAD dramatically (Hiller et al.,

1996). As a subpopulation of proteasomes are bound to the cytosolic face of ER membranes (Rivett et al., 1992; Palmer et al., 1996), it might be directly interacting with Sec61p retrotranslocon or with other proteins involved in the retrotranslocation process, or this subpopulation might even bind directly to the lipid bilayer (Newman et al., 1996; Lee et al., 2004; Kalies et al., 2005). Here, they might, at least partially, act as a 'driving force' for target proteins, removing them from the ER membrane. An attractive possibility is that membrane bound proteasomes could also be engaged in degradation of the cytosolic domains of integral membrane proteins prior to their extraction from the ER, for instance, degradation of Ubc6p and cytochrome P-450 (Roberts, 1997).

1.1.3 Protein folding and the unfolded protein response

Proper folding and maturation of nascent secretory proteins in eukaryotic cells is assisted by a large array of ER-resident chaperones and enzymes before they enter the subsequent compartments of the secretory pathway (Haigh and Johnson, 2002). The ER contains a specialized environment optimised for folding of newly imported polypeptides and posttranslational modifications to assume their native structures and to assemble into multimeric complexes. In the ER lumen, signal peptidase cleaves off the signal peptide from the nascent translocated protein; the oligosaccaryl-transferase complex (OST) carries out N-glycosylation; thiol-disulfide oxido-reductases such as protein disulfide isomerase (PDI) and Erp57 control disulfide bond formation. To guarantee delivery of only properly folded proteins to their site of action, chaperones located in the ER lumen play an essential role as well. On one hand, BiP/Kar2p, a member of the Hsp70 family, and other ER-resident chaperones bind to hydrophobic patches present in unfolded or partially folded proteins, and therefore help the nascent chain preserve the folding competence. On the other hand, BiP/Kar2p, which cooperates with soluble DnaJ-like proteins of the ER, Jem1p and Scj1p, facilitates the export of ERAD substrates to the cytosol by preventing their aggregation in the ER lumen. Thus, if proteins fail to fold correctly in the ER lumen, Kar2p may remain associated with them to retain them soluble in the ER. This prolonged association seems to supply a signal to the calnexin-calreticulin chaperones and to protein disulfide isomerase (PDI), which have a dual role: on one hand these chaperones help the target proteins assume their correct folding states, and on the other, they prevent progression of misfolded proteins through the secretory pathway (Kostova and Wolf, 2002; 2003).

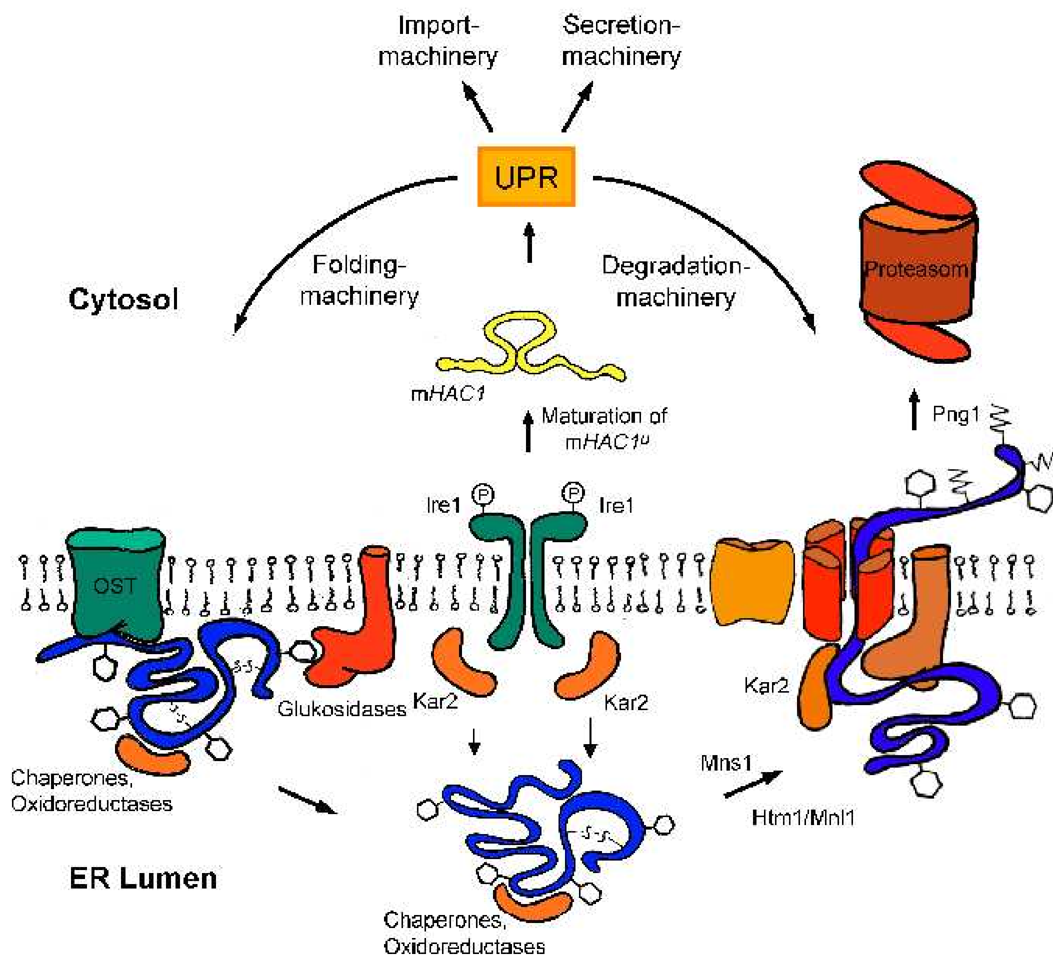


Figure 3. Unfolded protein response machinery See text (modified after R. Hitt, 2004)

Failure to fold proteins efficiently exposes the cell to the potentially toxic effects of unfolded proteins. As shown in Fig. 3, eukaryotic cells sense the accumulation of unfolded or misfolded proteins and respond by upregulating the synthesis of ER-resident chaperones via a signalling pathway called the unfolded protein response (UPR) (Sidrauski and Walter, 1997). In yeast, Ire1p is an ER transmembrane protein with activities of both, cytosol-facing serine/threonine kinase and RNase, localized in the ER/nuclear envelope, and acts as a sensor of the ER lumen. Both, unfolded proteins and the amino-terminal domain of Ire1p bind competitively to Kar2p. Abnormally high levels of unfolded proteins in the ER lead to depletion of free Kar2p and consequently dimerization of Ire1p. This conformational change transmits a signal across the membrane and activates nuclear kinase activity of Ire1p, which then induces a non-canonical splicing of mRNA encoded by the HAC1 gene, allowing synthesis of the Hac1p transcription factor. Hac1p upregulates expression of UPR target genes that contain an unfolded protein response element (UPRE) upstream of their promotor. This

results in an increase in the levels of ER resident proteins required for folding and quality control (Travers et al., 2000; Nock et al., 2001). UPR acts to reduce levels of misfolded proteins by enhancing their folding to the native state, by this promoting their transit to the distal secretory pathway. In yeast, UPR is induced by disrupting protein folding in the ER: simply by applying the strong reducing agent dithiothreitol (DTT), which prevents disulfide bond formation, or via the drug tunicamycin, which inhibits N-linked glycosylation. UPR and ER associated degradation are highly coordinated. Overexpression or accumulation of unfolded proteins due to the absence of components of the ER degradation machinery induces UPR (Knop et al., 1996a). In yeast, loss of function of components of both systems is lethal (Friedländer et al., 2000; Travers et al., 2000).

1.1.4 Recognition of malfolded proteins in the ER: The N-linked carbohydrate as sensor

N-linked glycosylation is one of the most common posttranslational protein modifications. The covalent attachment of hydrophilic oligosaccharides helps to solubilize the nascent protein chain and contributes to its proper folding, assembly and trafficking. When proteins are translocated across the ER membrane, core oligosaccharides of the Glc_3-Man_9-$GlcNAc_2$ structure are co-translationally attached to the side chains of asparagine residues within an Asn-X-Ser/Thr consensus sequence. As folding progresses, two of the outermost glucose residues of the N-linked glycan are trimmed by the glucosidases I and II. When the protein is properly folding, the remaining glucose residue is trimmed by glucosidase II, releasing a protein with the carbohydrate structure $Man_9GlcNAc_2$, leading to ER exit. In mammalian cells, a chaperone-mediated folding and retention mechanism in the ER lumen is especially well characterized. Two homologous lectins, calnexin and calreticulin , together with Erp57p, a member of the PDI family, bind to nearly all soluble and membrane glycoproteins imported into the ER. They specifically associate with glycoproteins that carry the monoglucosylated trimming intermediates (Glc_1-Man9$GlcNAc_2$) (Ellgaard et al., 1999; Hammond et al., 1994; Helenius et al., 1997) and are involved in a binding-release retention cycle driven by glucosidase II and UDP-glucose glycoprotein glucosyltransferase (UGGT) (Cabral et al., 2001). When, after trimming of the Glc1 residue by glucosidase II, folding of the protein is not completed, UGGT reglucosylates the carbohydrate chain and subsequent binding by calnexin/calreticulin allows a new round of folding. In case several rounds of folding do not result in a properly folded protein, α-1,2-mannosidase I cleaves the mannose 9 residue which finally leads to binding of the $Man_8GlcNAc_2$ structure by EDEM and delivering the associated malfolded protein to ERAD (Cabral et al., 2001; Jakob et al., 2001) (Fig. 4).

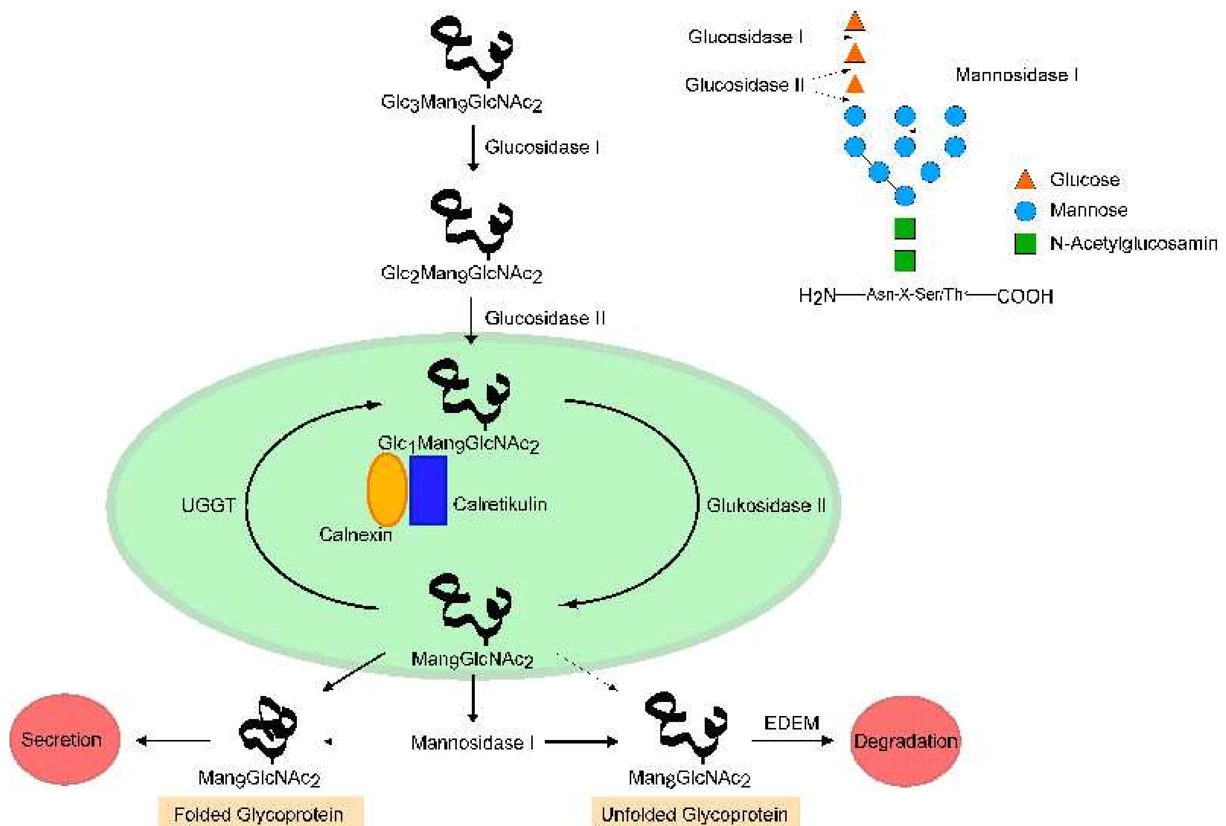


Figure 4. Timer of degradation of glycoproteins in mammalian cells. See text (modified after R. Hitt, 2004)

In yeast, the UGGT cycle is absent, therefore, carbohydrate trimming by glucosidase I and II and later by α-1,2-mannosidase I is believed to be the timer for protein folding and if not successful, for degradation (Kostova and Wolf, 2003). As in the case of mammalian cells, the trimmed $Man_8GlcNAc_2$ structure leads to binding of the malfolded protein to the lectin Htm1p, the yeast homologue of EDEM (Knop et al., 1996b; Plemper et al., 1998; Jakob et al., 2001; Nakatsukasa et al., 2001) and subsequent degradation. Model glycoprotein substrates used to study ER quality control in *S. cerevisiae* are a mutated vacuolar serine protease, carboxypeptidase yscY-S255R, known as CPY* (Finger et al., 1993), Pdr5*, a mutated form of the ATP-binding cassette transporter Pdr5 (Plemper et al., 1998) and mutated forms of proteinase yscA and Ste6p (Finger et al., 1993; loayza et al.,1998). In mammalian cells, well studied ER substrates are unassembled MHC class I heavy chain, mutant CFTR, mutant thyroglobulin and unassembled T cell receptor subunits. In addition, the concentration of Ca^{2+} ions in the ER has proved to be critical for the elimination of soluble CPY* but not for the integral membrane protein Pdr5*. It has been shown that glycosylated proteins of the ER are

retrotranslocated into the cytoplasm in a glycosylated state (Hiller et al., 1996; Wiertz et al., 1996). The discovery of de-N-glycosylated intermediates in the cytosol upon inhibition of the proteasome unravelled that cytoplasmic N-glycanase (Png1) preferentially removes N-glycans from misfolded proteins prior to proteasomal degradation (Suzuki et al., 2000, 2001; Misaghi et al., 2004). Even though so much is known about the components required for recognition and elimination of misfolded glycoproteins, till now, the mechanism of the ER quality control processes of carbohydrate-dependent targeting, retention and degradation as well as recognition of non-glycosylated proteins are still poorly understood.

1.1.5 Physiological regulation via the ER quality control pathway

Yeast and mammalian cells utilize the ER quality control machinery to physiologically regulate the levels of some ER proteins and enzymes. One of the best-studied examples is the downregulation of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-R), a key regulatory enzyme in sterol synthesis. Farnesylpyrophosphate is the primary signal for HMG-R2 degradation in yeast (Hampton and Bhakata, 1997) as well as for degradation of the mammalian homologue HMG-R (Meigs and Simoni, 1997). Der3p, Hrd3p and Ubc7p were identified as necessary elements for degradation of HMG-R2 in yeast and degradation occurs via the ubiquitin-proteasome system (Hampton et al., 1996; Bays et al., 2001; Gardner et al., 2000). By virtue of signals resulting in allosteric changes from the mevalonate pathway, the conversion of the HMG-R is regulated from a stable protein into a quality control substrate (Cronin et al., 2000).

1.1.6 Protein quality control and disease

Inefficient folding, unbalanced subunit synthesis, or mutations in secretory proteins result in the failure of translocated polypeptides to assume their correct conformation. A defect in ER degradation leads to accumulation of misfolded proteins in the ER. In recent years, formation of protein aggregates has been shown to be a main feature in the pathogenesis of several diseases, such as BSE, Creutzfeldt-Jakob disease and other prion diseases (reviewed in Kostova and Wolf, 2002). A ‘‘hyperactive‘‘ ER-degradation process leads to the severe disease of cystic fibrosis (Jensen et al., 1995; Ward et al., 1995) and to immune suppression (Wiertz et al., 1996; for review see Kostova and Wolf, 2002). Consequently, obtaining a detailed mechanistic insight into the dislocation and elimination process of such malfolded proteins is of great interest for the understanding of the molecular basis of such diseases.

1.2 Purpose of the study

A variety of studies indicate that the retrograde transport of ER-resident misfolded proteins depends on Sec61p (Wiertz et al., 1996a; Pilon et al., 1997; Plemper et al., 1997,1998; Bebök et al., 1998; Zhou and Schekman, 1999). The ERAD associated components identified by genetic analyses and biochemical methods are, in some cases, shown, and in others, believed to interact with Sec61p, the core protein of the import channel of secretory proteins into the ER (Sommer and Wolf, 1997; Zhou, et al., 1999; Johnson, et al., 1999; Johnson and Haigh, 2000). Various studies propose that the composition of the retrograde export channel will differ substantially from that of the import channel (Wiertz, et al., 1996; Pilon, et al., 1997; Römisch, 1999; Plemper et al., 1997,1999; Ye et al., 2004; Lilley and Ploegh, 2004). In order to get direct biochemical proof for the composition of the export channel and to clarify interactions among known components as well as to examine the possible role of the Sec61p in retrotranslocation, this study was undertaken. Appropriate methods as Blue Native electrophoresis and co-immunoprecipitation were adapted to the posed problems and applied in this study, in the hope of contributing to a better understanding of the mechanisms of retrotranslocation of malfolded proteins from the ER back to the cytoplasm.

2. Materials and methods

2.1 Materials

2.1.1 Saccharomyces cerevisiae strains

Strain	**Genotype**	**Reference**
BY4743	*MAT**a**/MATα MET15/Δmet15 LYS2/Δlys2 Δura3/Δura3 Δleu2/Δleu2 his3-1/his3-1*	EUROSCARF, Frankfurt
BY4743*Δhrd3*	BY4743 *Δhrd3::KAN*^{R}	EUROSCARF
MHY1366	*MAT*a his3-*Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 prc1-1*	Swanson *et al.*, 2002
MHY1651	*MAT*α his3-*Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 prc1-1Δdoa10::HIS3*	Swanson *et al.*, 2002
W303-1B	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	Chiang and Schekman, 1991
W303-1C	W303-1B *prc1-1*	Knop *et al.*, 1996a
W303-ΔC	W303-1B *MAT*a *Δprc1::LEU2*	R. Plemper
W303-CΔD	W303-1C *Δder1::HisG*	Knop *et al.*, 1996a
W303-CtD	W303-1C *DER1::HA*	Knop *et al.*, 1996a
YCT519	W303-1C *MAT*a *Δubc1::HIS3 Δubc7::LEU2*	C. Taxis
YJB 005	W303-1B *MATα prc1-1 Δhrd3::HIS3*	Plemper *et al.*,1999
YJB 009	W303-1B *MATα prc1-1 Δder3::HIS3*	Bordallo *et al.*,1998
YWO0433	W303-1B *MATα prc1-1 Δder3::HIS3 Δhrd3::HIS3*	Bordallo *et al.*,1998
YXL003	W303-1C *Δder3::HIS3 Δubc1::HIS3 Δubc7::LEU2*	This work
YXL005	W303-1B *MATα Δprc1::LEU2 Δubc1::HIS3 Δubc7::LEU2*	This work
YXL009	W303-*Δ*C *MATα Δder3::HIS3*	This work
YXL023	W303-*Δ*C *MATα Δhrd3::HIS3*	This work
YXL011	W303-1C *MAT*a *Δhrd3::HIS3 Δubc1::HIS3 Δubc7::LEU2*	This work
YXL013	W303-1C *MATα Δder3::HIS3 Δhrd3::KAN*^{R} *Δubc1::HIS3 Δubc7::LEU2*	This work
YCT519-1	W303-1C *MATα Δubc1::HIS3 Δubc7::LEU2*	This work (R.Hitt)

YXL026	W303-*Δ*C *MAT*α *Δder3::HIS3 Δhrd3::HIS3*	This work
YXL035	W303-1B *MAT*α *Δprc1::LEU2Δhrd3::HIS3 pre1-1*	This work
YXL036	W303-1B *MAT*a *Δprc1::LEU2 pre1-1*	This work
YXL038	W303-1B *MAT*α *Δprc1::LEU2Δhrd3::HIS3 pre1-1 NPL4::13myc::*KAN^R	This work
YXL039	W303-1B *MAT*α *Δprc1::LEU2Δhrd3::HIS3 NPL4::13myc::*KAN^R	This work
YXL040	W303-1B *MAT*a *Δprc1::LEU2 NPL4::13myc::*KAN^R	This work

2.1.2 Escherichia coli strains

Strain	**Genotype**	**Reference**
DH5α	*supE44* Δ*lac* U169 (ϕ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*69 *thi*-1 *relA*1	Hanahan 1983
XL2-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F'*proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tet^r) Amy Cam^r]	Stratagene

2.1.3 Plasmids

Plasmid	**Insertion**	**Reference**
pCT42	pRS316/P_{TDH3}-CPY*::3HA	C. Taxis
pCT51	pRS316/P_{CUP1}::CPY*::3HA	C. Taxis
pCT67	pRS316(URA3, HIS3)/P_{TDH3}::CTG*	C. Taxis
pHIT304	pRS316/P_{CPY}::CPY*DHFR$_{WT}$	R. Hitt
pHIT304suc	pRS316/P_{CPY}::SS_{SUC2}::CPY*DHFR$_{WT}$	R. Hitt
pHIT324	pRS316/P_{CPY}::CPY*DHFR$_{mut}$	R. Hitt
pHIT324suc	pRS316/P_{CPY}::SS_{SUC2}::CPY*DHFR$_{mut}$	R. Hitt
pHIT341	pRS316/*prc1-1*	R. Hitt
pHIT344	pRS316/CPY::CPY*Rhodanese	R. Hitt
pHIT348	pRS316/CPY::CPY*BPTI	R. Hitt
pHIT349	pRS316/CPY::CPY*BPTI::3HA	R. Hitt
pHIT360	pRS316/CPY::CPY*SUC2	R. Hitt
pJK59	pRS316/Sec63::GFP	Prinz et al., 2000
pMA1	pRS316(URA3, HIS3)/P_{TDH3}::CTG*	C. Taxis
pRS315-DER3-3HA	pRS315/DER3::3HA	P. Deak
pTS03	pRS306/*pre1-1*	H.Wolfgang

pTX190a	pRS316/P_{CUP1}::CPY*	C. Taxis
pXL3140	pRS314/3HA::Hrd3	this work
pXL3141	pRS314/P_{CUP}::CPY*	this work
pXL3161	pRS316/P_{SUC2}::ssSuc2::Suc2CPY*::T_{CPY}	this work
pXL3162	pRS316/P_{CPY}::ssSuc2::CPY*rGA215	this work
PXL3163	pRS316/P_{CPY}::ssSuc2::CPY*rGA215::3HA	this work
pXL3164	pRS316/P_{TDH3}::CPY*rGA215	this work
pXL4241	pRS424/P_{SUC2}::ssSuc2::Suc2CPY*::T_{CPY}	this work
pXL4242	pRS424/CPY*GFP	this work
pXL4261	pRS426/P_{SUC2}::ssSuc2::Suc2CPY*::T_{CPY}	this work
pYS14	pRS316/3HA::Hrd3	Saito et al., 1999
pZK102	pRS316/P_{CPY}::CPY*rGA215	K. Zlatka
pZK129	pRS316/P_{CPY}::CPY*rGA215::3HA	K. Zlatka
YCP315-*der3CS*-3HA	YCP(CEN, LEU2)/*der3C399S::3HA*	J. Bordallo

2.1.4 Antibodies

Antibody	**Dilution**	**Reference**
Rabbit α-Cdc48	1:200 for IP 1:10000 for western	F. Madeo *et al.*, 2000
Rabbit α-Cdc48	1:500 for IP 1:10000 for western	T. Sommer
Rabbit α-CPY	1:200 for IP	Finger *et al.*, 1993
Rabbit α-Cue1	1.2500 for western	
Rabbit α-Der3	1:2500 for western	J. Bordallo *et al.*, 1998
Rabbit α-Flag	1:500 for IP 1:5000 for western	Sigma
Rabbit α-Kar2	1:10000 for western	Rose *et al.*, 1989
Rabbit α-Sec61	1:500 for IP 1:10000 for western	Biederer *et al.*, 1996
Rabbit α-HA	1:500 for western	Sigma, Deisenhofen
Mouse α-CPY	1:10000 for western	Mol. Probes
Mouse α-GFP	1:10000 for western	Babco/Covance, USA

Mouse α-HA-Epitope	1:500 for IP 1:10000 for western	Babco/Covance, USA
Mouse α-myc-Epitope	1:500 for IP 1:5000 for western	Calbiochem
HRPO conjugated Goat α-Mouse	1:10000 for western	Dianova, Hamburg
HRPO conjugated Rabbit α-Mouse	1:10000 for western	Sigma, Deisenhofen

2.1.5 Enzymes

Restriction endonucleases used in this study were provided either by Roche or by New England Biolabs (NEB).

Name	**Resource**
Creatine Phosphate Kinase	Boehringer/Roche, Mannheim
Deep® Vent-DNA-Polymerase	NEB
Endoglycosidase H	NEB
Glusulase	Perkin Elmer Inc., Boston, MA, USA
Oxalyticase	Enzogenetics, Corvallis, OR, USA
Protease K	Sigma, Deisenhofen
PWO-DNA-Polymerase	Boehringer/Roche, Mannheim; Peqlab, Erlangen
RNAse A	Sigma, Deisenhofen
T4-DNA-Ligase	Boehringer/Roche, Mannheim; NEB, Beverly, USA; Gibco/Invitrogene, Karlsruhe
Taq-DNA-Polymerase	Boehringer/Roche, Mannheim

2.1.6 Kits

Name	**Resource**
QIAEX II Gel extraction Kit	Qiagen, Hilden
QIAprep Spin Miniprep Kit	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
ECL-Western Detection Kit	Amersham-Pharmacia, Little Chalfont,GB
HMW Native Marker Kit	Amersham-Pharmacia, Little Chalfont,GB
Lumi-Light Western Blotting Substrate	Boehringer/Roche, Mannheim
Silver Staining Kit	Amersham-Pharmacia, Little Chalfont,GB

2.1.7 Chemicals

Unless otherwise indicated, the chemicals used in this study were provided by the companies Genaxxon, Merck, Roth, Roche and Sigma.

Name	**Resource**
Acrylamide	Serva
ε-aminocaproic acid	Sigma
All-Blue Protein Marker	Bio-Rad, Hercules, CA, USA
ATP	Boehringer/Roche, Mannheim
Bis-acrylamide	Serva
Bis-Tris	Sigma
Complete^{TM} Inhibitor-Cocktail	Boehringer/Roche, Mannheim
Coomassie G250	Serva
Creatine phosphate	Boehringer/Roche, Mannheim
Cycloheximide	Sigma, Deisenhofen
Deoxy-Big-CHAP	Calbiochem
DNA molecular weight marker	NEB; Gibco/Invitrogene, Karlsruhe
Digitonin	Calbiochem
n-Dodecyl-β-D-maltoside	Calbiochem
ECL Chemiluminesense^{TM} film	Amersham-Pharmacia, Little Chalfont, GB
5-FOA	Toronto Research Chemicals Inc., Canada
Gel-Blotting Paper GB001-003	Schleicher & Schuell, Dassel
Nitrocellulose Membrane Biotrace^{TM} NT 0.45µm	Pall Corporation, Ann Arbor, MI, USA
Protein A-Sepharose CL-4B	Amersham-Pharmacia, Little Chalfont, GB
Protein G Plus/Protein A agarose	Calbiochem-Oncogene, Darmstadt
Protogel 49,5%	Calbiochem
PVDF-Immuno-Blot-Membrane	Bio-Rad, Hercules, CA, USA
See-Blue Protein Marker	Invitrogene
Tunicamycin	Sigma
10% Triton X-100	Calbiochem

2.2 Methods

2.2.1 Growth conditions of model organisms

2.2.1.1 Saccharomyces cerevisiae

The standard media were applied in this study for inoculation of yeast strains (Ausubel, 1992; Guthrie and Fink, 1991). Yeast were usually grown at 30°C either on complete medium (YPD) (1% (w/v) Bacto® yeast extract, 2% (w/v) Bacto® peptone, 2% (w/v) glucose, pH 5.5) or on synthetic medium (SD) (0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose, pH 5.6) containing 0.3 mM adenine, 0.4 mM tryptophan, 1 mM lysine, 0.3 mM histidine, 1.7 mM leucine, and 0.2 mM uracil (final concentration) (complete SD medium). Cells were grown to logarithmic phase (1-3 OD_{600} units/ml) at 30°C on a rotary shaker, unless otherwise indicated. Yeast transformants carrying plasmids were grown in SD medium lacking either leucine, or uracil, or tryptophan, or uracil and tryptophan depending on the selection marker genes (*LEU2*, or *URA3*, or *TRP4* respectively) present on the plasmids (selective SD medium). In order to induce the expression of CPY* under the *CUP* promoter, 100 µM of $CuSO_4$ as final concentration was added into selective SD medium when the cells were grown to 0.6-0.8 OD units/ml. The culture has to be incubated at 30°C for another 3-5 hrs (i.e. for one doubling of cell number).

Solid medium contained 2% (w/v) Bacto® Agar in addition to the YPD or SD medium components. Plasmid shuffle in yeast transformants carrying one plasmid with the *URA3* encoding gene as a marker was induced by incubation on SD plates containing 0.5 mM uracil (final concentration) and 1mg/ml fluoro-orotic acid (5-FOA) in addition, resulting in the loss of the plasmid carrying the *URA3* marker. Selective growth of yeast cells carrying a gene deletion marked by the kan^r gene for geneticin resistance was performed by incubation on YPD plates containing 0.2 mg/ml geneticin (G-418 sulphate).

The sporulation of diploid cells was induced by incubation on YPD plate at 30°C overnight and then on sporulation plates (1% (w/v) potassium acetate and 2% (w/v) Bacto® Agar) at 25°C for 3-4 days.

Yeast strains were permanently stored in 15% Glycerol at −80°C.

2.2.1.2 Escherichia coli

Cells were propagated in LB medium (0.5% (w/v) yeast extract, 1% (w/v) Bacto® trypton, 0.5% (w/v) NaCl, pH 7.5) or SOC medium (2% (w/v) Bacto® trypton, 0.5% (w/v)

Bacto® yeast extract, 0.4% (w/v) glucose, 2mM NaCl, 0.25mM KCl, 1mM $MgCl_2$, 1mM $MgSO_4$) and grown at 37°C. Cells transformed with plasmids carrying the gene *amp*^{r} for ampicillin resistance as selection marker were propagated in LB medium containing 0.1mg/ml ampicillin.

E. coli strains were permanently stored in 30% Glycerol at −80°C.

2.2.2 Genetic methods

2.2.2.1 Yeast transformation

Standard lithium acetate methods for plasmid transformation into yeast were used according to Soni et al., 1993. The transformants were selected for URA^+, LEU^+ or TRP^+ prototrophy dependent on plasmids.

Transformation of yeast with PCR fragment containing *kan*^{r} was performed according to Achim *et al*., 1995. After Ethanol precipitation, PCR product was resuspended in 10μl TE buffer (10mM Tris-HCl, 1mM EDTA) and transformed into freshly prepared yeast competent cells with lithium acetate method. After heat shock at 42°C for 20min, the cells were resuspended in 10ml YPD media and the culture was incubated at 30°C with shaking for another 3hrs. Then the cells were resuspended and spread on YPD plates containing 200mg/l geneticin (G418) and incubated at 30°C for 1-2 days. Bigger colonies from replica plates were selectively streaked on new YPD-G418 plates for three times to exclude probability of false positive colonies.

2.2.2.2 Mating type test

The mating type of yeast strain can be tested through two test strains YP312 (mating type a) and YP320 (mating type α), which contain an auxotrophic marker *his1-123* mutation and can not grow by itself on MV plate (0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose, pH 5.5) without any supplementary amino acids. Only diploid cells can grow on such kind of plate after YP312 or YP320 is mated with a strain which not only to be mating type matched, but also has to contain a complementary mutation like *his3-11* in W303-1B isogenic background. For instance, if an unknown strain is able to mate with YP312 and is not able to mate with YP320, its mating type should be α. Contrarily, its mating type should be a.

2.2.2.3 Growth test

For temperature sensitive growth test of yeast, 1OD of cells were resuspended in 1ml sterile dH_2O and diluted into 10^{-1}~10^{-4}. 5µl of cells were dropped on YPD plate. The plates were incubated either at indicated temperature for 2-3 days.

2.2.2.4 Construction of yeast strains

2.2.2.4.1 Deletion through mating

The strains YXL009 (*Mat α, Δprc1::LEU2, Δder3::HIS3)*, YXL023 (*Mat α, Δprc1::LEU2, Δhrd3::HIS3)*, YXL026 (*Mat α, Δprc1::LEU2, Δder3::HIS3, Δhrd3::HIS3)* were generated by cross mating W303ΔC (*Mat a*, *Δprc1::LEU2*) with YWO0433 (*Mat α, Δder3::HIS3, Δhrd3::HIS3, prc1-1*). The strain YXL005 (*Mat α, Δprc1::LEU2, Δubc1::HIS3, Δubc7::LEU2*) was generated by cross mating W303ΔC (*Mat a, Δprc1::LEU2*) with YCT519-1 (*Mat α, Δubc1::HIS, Δubc7::LEU2, prc1-1*). The strain YXL003 (*Mat α, Δder3::HIS3, Δubc1::HIS3, Δubc7::LEU2, prc1-1*) was generated by cross mating YJB009 (*Mat a, Δder3::HIS3, prc1-1*) with YCT519 (*Mat a, Δubc1::HIS3, Δubc7::LEU2, prc1-1*). The strain YXL011 (*Mat a, Δhrd3::HIS3, Δubc1::HIS3, Δubc7::LEU2, prc1-1*) was generated by cross mating YJB006 (*Mat α, hrd3::HIS3, prc1-1*) with YCT519 (*Mat a, Δubc1::HIS3, Δubc7::LEU2, prc1-1*). In brief, haploid cells with different mating type (a or α) were cross-streaked and mated on a YPD plate at 25°C for 4-8 hrs. After the zygotes were observed under microscope, the single zygote was picked out and put on a new YPD plate by a microneedle under the microscope, following incubation at 30°C for 2-3 days. Then heterozygous diploid cells were sporulated on a 2% potassium acetate plate at 25°C for 3-4 days. Tetrads dissection were performed in 200µl sterile dH_2O containing 1.5% Glusulase at room temperature for 3min and continuously at 4°C for 1hr after 800µl sterile DH_2O was added. Tetrads were then separated under the light microscope using a microneedle coupled to a micromanipulator. The complementation analysis for requirement of either histidine-, leucine- or histidine- and leucine were performed after four spores come out on YPD plate. In addition, a mating type test for the four spores were performed to make sure they are two to two meiotic segregants. Finally, the selected spores were analysed by PCR and Western blotting.

2.2.2.4.2 PCR-based gene disruption through homologous recombination

Deletion of gene *HRD3* was performed via the methods of PCR-based gene disruption through homologous recombination (Wach et al., 1994; Orr-Weaver et al., 1981; Orr-Weaver

and Szostak, 1983). In order to produce the tetra deletion strain YXL013 (*Δder3::HIS3, Δhrd3::KanMX4^R, Δ ubc1::HIS3, Δubc7::LEU2*), genomic DNA of in the EUROSCAF strain collection BY4743 *Δhrd3::KanMX4^R* was isolated, which served as the template in a subsequent PCR using the oligonucleotide 5´ ATT GAA AAC TTT CGG GTA ATG C-3´ and 5´ACG AAC TCG CAC TTC AGC –3´ as sense and anti-sense primers. The resulting 1.6kb PCR product, which contains separately ~100bps nucleotides of both upstream and downstream of *HRD3* gene sequence and a *kanMX4* selectable marker, was transformed directly into haploid cells of YXL003 (*Mat α, Δder3::HIS3, Δubc1::HIS3, Δubc7::LEU2, prc1-1*). Deletion of the gene *HRD3* from YXL003 was verified by colony PCR.

2.2.2.4.3 Mutation through an integrative plasmid

In order to introduce *pre1-1* allele into W303 isogenic background, pop-in and pop-out method was applied according to Seherer and Dans, 1979. pTS03, which contains a *pre1-1* allele in *URA3*-marked integrative vector YIp5, was linearized by *SacI* and transformed into W303ΔC (*Mat a, Δprc1::LEU2)* and YXL023 (*Mat α, Δprc1::LEU2, Δhrd3::HIS3)* to generate YXL036 (*Mat a, pre1-1, Δprc1::LEU2)* and YXL035 (*Mat α, pre1-1, Δprc1::LEU2, Δhrd3::HIS3)* respectively. Descendants which by recombination have integrated plasmid sequence were selected by growth on URA$^-$ plates and then applied with a 5'-fluoroorotic acid selection, which leads to loss of *URA* and *PRE1* sequence. The integration was proved by temperature sensitive growth (38.5°C) test and chymotrypsin-like activity assay of the proteasome (Heinemeryer et al., 1997).

2.2.3 Molecular biological methods

All DNA manipulations were carried out by standard techniques (Sambrook et al., 1989; Ausubel, 1992). Enzymes were used as suggested by the provider. *E. coli* cells were transformed by heat shock. The DNA was purified via QIAEX II Gel extraction Kit, QIAprep Spin Miniprep Kit and QIAquick PCR Purification Kit. The PCR-amplified DNA fragments were sequenced by Biolux, Stuttgart-Vaihingen or GAGC, Konstanz.

2.2.3.1 Isolation of yeast genomic DNA

10 ml YPD of yeast overnight culture were pelleted by centrifuging at 3000 × g for 3min at room temperature. The culture medium were removed by aspiration and the pellet were resuspended in 0.5ml dH_2O in a microcentrifuge tube. Freshly prepared 200µl of breaking buffer (10mM Tris-HCl (pH 8.0), 1mM EDTA, 0.1M NaCl, 1%SDS, 2% TritonX-100), 200µl of Phenol:Chloroform:Isopropanol (24:25:1) and 200µl of acid-washed glass

beads were added to the tube, the organic mixture was vortexed for 3-4min. After adding 200µl TE buffer (50mM Tris-HCl (pH8.0), 2mM EDTA), the tube is centrifuged at 20,000× g for 3min and the upper aqueous phase was transferred to a fresh microtube. The DNA was precipitated with ethanol for 30min at –20°C. The precipitate of nucleic acids was recovered by centrifugation at 20,000× g for 10min at 4°C. After removal of supernatant by aspiration, the pellet was dried in the air for 15min, redissolved in 0.4ml of TE plus 3µl 100mg/ml RNase and incubated at 50°C for 45min. After being recovered by centrifugation at 20,000× g for 5min at RT, the pellet containing genomic DNA was washed once with 1ml of 70% ethanol plus 10µl 3M Sodium acetate, air dried and resuspended in 50µl of TE.

2.2.3.2 Colony-PCR of Yeast cells

A single colony of yeast strain was picked and transferred into PCR microtube by a toothpick. The cells were opened in microwave for 1min by using a power of 800W. Then the cells were resuspended in 40µl of PCR reaction mixture (32µl H_2O, 4µl 10 × Taq PCR buffer, 1µl 25mM MgCl2, 1µl 10mM dNTPs, 0.4µl of each 100 pmol/L primer). After one cycle of "hot start" at 95°C for 5min, 0.7µl of *Taq* polymerase was added and 25 PCR cycles were carried out.

2.2.3.3 Construction of plasmids

pXL4261 was constructed by using a 2-step PCR method. PCR was carried out to amplify the *SUC2* gene using yeast chromosomal DNA of W303-1C as template (reaction 1) with the sense primer 5´-CC ATC GAT ACG CGT AGC GTT AAT CG-3´ containing *Cla*I digestion site and anti-sense primer 5´-CGG TCT TTG CAA TGA GAT CATATG TTT TAC TTC CCT TAC TTG G-3´ containing an extra Nde I digestion site in the middle of sequence. The DNA fragment corresponding to *prc1-1* (CPY*) was amplified from pHit341 containing this gene (reaction 2) with the sense primer 5´-CCA AGT AAG GGA AGT AAA A CATATG AT CTC ATT GCA AAG ACC G-3´ containing an extra *Nde* I digestion site in the middle of the sequence and anti-sense primer 5´- CGG AAT TCA TTG TAC TTA CAA ACT CG-3´ containing *Eco*RI digestion site. A third PCR, using the products of reaction 1 (*SUC2*) and reaction 2 (*prc1-1*), was performed to obtain the fusion product *SUC2-prc1-1*. This fusion product was cloned at the *Eco*RI-*Cla*I sites of yeast 2µ vector pRS426. DNA sequencing confirmed that no mutations were introduced, due to PCR errors, within the coding sequence of SUC2-CPY*. For further studies, SUC2-CPY* fragment was subcloned into *Eco*RI-*Cla*I sites of a yeast centromeric vector pRS316 to generate pXL3161.

To generate a *HA_3-HRD3* expressing plasmid with *TRP4* marked gene sequence, a *Kpn*I-*Sac*I fragment (3324bps) containing *HA_3-HRD3* from pYS14 was subcloned into pRS314 resulting in pXL3140.

pXL3141 was generated by subcloning a *Kpn*I-*Sac*I fragment (2797bps) containing *prc1-1* under CUP promoter from pTX190a into pRS314.

A *Bsu36I-ClaI* fragment (1341bps) from pHit304suc containing *CPY* promoter, signal sequence of *SUC2* and partial gene of *CPY* was subcloned into pZK102 to produce pXL3162. A *Bsu36I-XhoI* fragment (1341bps) from pHit304suc containing *CPY* promoter, signal sequence of *SUC2* and partial gene of *CPY* was subcloned into pZK129 to produce pXL3163. A *Bsu36I-EcoRI* fragment (2129bps) from pCT67 containing *TDH3* promoter, signal sequence of *CPY* and partial gene of *CPY* was subcloned into pZK102 to produce pXL3164.

2.2.4 Biochemical methods

Quantification of DNA was done by measurement of OD260 and OD280.

Quantification of Protein was measured by standard Bradford test.

2.2.4.1 Alkaline lysis of yeast whole cell extracts

2-3 OD_{600} units of yeast cells were incubated with 30mM NaN_3 and harvested at 20,000 × g for 1 min, the pellet was resuspended in 1ml of dH_2O. After the cells were lysed on ice for 10 min using 150µl lysis buffer (2M NaOH, 7.5% β-ME), 150µl of 55% TCA was added and the cells were incubated on ice for another 10min. The precipitated proteins were centrifuged at 20,000 × g for 10min at RT and the pellet was resuspended in 100µl Urea Sample Buffer (8M Urea, 200mM Tris-HCl, pH 6.8, 0.1mM EDTA, 5%(w/v) SDS, 1.5%(v/v) *β*-ME) and mixed on a multivortex at 37°C for 15-20min. After a centrifugation at 20,000 × g for 5min at RT, 10-15µl of the supernatant was subjected to a SDS-PAGE (see 2.2.4.3).

2.2.4.2 Silver staining and Gel Drying

Denaturing SDS-polyacrylamide gel was visualized by silver staining which was performed according to manufacturer's instruction of Kit (see 2.1.6). All PAGE-Gels were dried at 65°C by a Gel-dryer under vacuum.

2.2.4.3 SDS-PAGE and Western Blotting

Denaturing SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to Lämmli, 1970. SDS polyacrylamide gradient gels of 1.5mm thickness of 5-16% or 5-12.5% were made by using a Gradient Former (Bio-Rad). Either Mini- or Midi- Protein system (Bio-Rad) was adopted.

After migration on SDS-PAGE gels, protein were electro-transferred to nitrocellulose or PVDF membranes for 60-90min by using a semi dry transfer system (Hoefer Pharmacia Biotech, San Francisco, CA). Equal loading per lane was confirmed by a brief staining with *Ponceau* S. Pre-incubation, antibody incubations and washes were performed in PBST (16mM Na_2HPO_4, 4mM NaH_2PO_4, 100mM NaCl, 0.1%Tween). For western analysis, detection of the specialized proteins was performed using the respective antibody as indicated. Immunodetection was carried out according to the manufacturer's protocol from ECL Chemiluminescence detection Kit (Amersham Biosciences) or Lumi-Light Western substrates Kit (Roche). When it was required, the Nitrocellulose membrane or PVDF membrane was reprobed with other antibodies after being incubated with harsh stripping buffer (62.5mM Tris-HCl, 2% (w/v) SDS, 100mM β-ME) at 60°C for 25min with occasional agitation.

2.2.4.4 Cycloheximide chase analysis

Degradation of targeted proteins were evaluated by treatment of logarithmic phase strains (OD_{600} 1.0-1.5) with Cycloheximide (100µg/ml) to halt protein synthesis. Cells were removed at the indicated time points and the reactions were stopped by the addition of 30 mM NaN_3 solution. Afterwards, either alkaline lysis (see 2.2.4.1) was performed (2.5-3.0 OD_{600}/time point) or crude microsomal membranes (50-80 OD_{600}/time point) were natively isolated and solubilized (see 2.2.4.7), dependent on different experimental purpose indicated. The degradation of targeted proteins were detected by immunoblots (see 2.2.4.3).

2.2.4.5 Tunicamycin treatment experiment

N-Glycosylation of Glycoproteins was inhibited by treatment of logarithmic phase growing 0.8-1.0 OD_{600} Cells with Tunicamycin (50µg/ml) in *vivo*. 2.5 OD_{600} units of cells from indicated strains were subjected to alkaline lysis (see 2.2.4.1) at indicated time points and immunoblots for targeted protein were followed (see 2.2.4.3).

2.2.4.6 Preparation of yeast spheroplasts

50-500 OD_{600} units of logarithmic phase yeast cells OD_{600} (1.0-3.0) were collected and washed once with 15mM Sodium azide, then cells were resuspended and incubated in TS buffer (0.1M Tris/Sulphate, pH 9.4, 20mM DTT buffer) in 30°C water bath for 10-15min. Cells were then resuspended in 1-2 ml of Sorbitol buffer (1M Sorbitol, 25mM Na_2HPO_4, 25mM NaH_2PO_4) with addition of oxalyticase (50µg/ml in a final concentration) and incubated 30min in 30°C water bath with slightly agitation. Efficiency of spheroplasting were controlled under the light microscope and harvested carefully with a gentle centrifugation at $500 \times g$, for 5min at 4°C.

2.2.4.7 Preparation and solubilization of crude microsomal membranes

Unless otherwise stated, all the steps were carried out at 4°C. Spheroplasts were resuspended at 100OD_{600} units/ml in ice cold PS200 buffer (200mM Sorbitol, 20mM Pipes (pH 6.8), 5mM $MgCl_2$, 1mM DTT) and equal amount of ice cold B buffer (250mM Sorbitol, 20mM Hepes/KOH (pH 7.4), 50mM KOAc, 5mM $MgCl_2$, 1mM DTT, 1mM EDTA) containing 1mM PMSF, 1mM protease inhibitor cocktail (Roche), 1mg/ml Pepstatin A, 1mg/ml Antipain, 1mg/ml Leupeptin and 1mg/ml Chymostatin respectively, and lysed by using glass homogeniser for 20 strokes. The homogenates then was centrifuged at 1500 × g for 5min at 4°C for twice to remove unlysed spheroplasts. The supernatant containing vesicles were fractionated by centrifugation for 1hr at 48,000× g at 4°C. The pellet of crude microsomal membrane was washed once with ice cold B buffer and resuspended in ice-cold membrane storage buffer B2 (20mM Hepes/KOH (pH 7.4), 50mM KOAc, 500mM EACA, 20% (w/v) Glycerol, 5mM $MgCl_2$, 1mM DTT, 1mM EDTA, 1mM PMSF). After the protein concentration was defined according to Bradford, (1976), the microsomal membranes were frozen directly by liquid nitrogen and stored at −80°C.

The washed membrane suspension was solubilized by adding solubilization buffer B1 (20mM HEPES/KOH, pH7.4, 50mM KOAc, 500mM ε-aminocaproic acid (EACA), 10% (w/v) Glycerol, 2mM EDTA, 1mM DTT, 1mM PMSF) and a defined volume of 5% Digitonin / 1% Triton X-100 / 10% Deoxy-BigCHAP / 5% DDM to a series of different final concentration of detergents as indicated, respectively. After incubating for 1hr in cold room with Top-to-End rotation, insoluble material was removed by ultra centrifugation for 30min at 75000rpm (200,000 × g) at 4°C in a Beckman TLA 120.2 (Beckman Instruments, Palo Alto, CA). The solubilized supernatant was freshly in use.

2.2.4.8 Endo-H in vitro digestion

40 OD600 log phase growing cells were harvested , spheroplasted (see 2.2.4.6), and lysed with 1ml denaturing Hepes lysis buffer (50mM Hepes/KOH (pH 7.5), 140mM NaCl, 1mM EDTA, 2% (v/v) Triton X-100, 0.5% (w/v) Sodium deoxycholate, 0.1% SDS, 1mM freshly added PMSF and 0.5% (v/v) Complete™ protease inhibitor cocktail, Roche) following an incubation on ice for 20min. The lysate was separated by centrifugation for 15min at 20,000 × g at 4°C. The supernatant was incubated for 2hrs with 5µl of polyclonal Rabbit anti-CPY by using a Top-to-End rotator in cold room. Then the mixture was equally divided into two tubes. Each tube was added with 5.6mg Protein A Sepharose® CL-4B in 80µl Hepes lysis buffer and incubated on the rotator in the cold room for another 1 hr. Then the Sepharose

beads were collected and washed gently with 1.4ml Hepes lysis buffer for 5 times and 1 time with 1.4ml potassium phosphate washing buffer (50mM Potassium phosphate (pH 5.5), 0.02% (w/v) SDS, 1mM PMSF, 0.5% (v/v) Complete^{TM} protease inhibitor cocktail) by centrifugation for 20 seconds at 1000 × g at RT. After the Sepharose beads were resuspended in 25µl potassium phosphate washing buffer containing additional 0.7% (v/v) β-ME. 2µl Endoglycosidase H was added into one of the aliquots. Then both tubes were incubated at 37°C for 1hr and the digestion was stopped by adding 25µl Urea sample buffer containing 1.5% β-ME and heated for 5min at 95°C. Before being subjected to the SDS-PAGE, the samples were cleared by centrifugation for 5min at 20,000 × g at RT.

2.2.4.9 Protease K protection experiment

All steps were carried out on ice or at 4°C. The spheroplasts were prepared as described in 2.2.4.7. Afterwards, the spheroplasts were lysed with ice-cold PS200 buffer just with pipettes to keep vesicles be intact. The supernatant from centrifugation at 1500 × g were split into 250 µl aliquots and were either mixed (1) with 250 µl of PS200 buffer with 0.1 mM PMSF or (2) with 250 µl PS 200 buffer with 0.1mM PMSF containing 0.2 mg/ml Proteinase K or (3) with 250 µl of PS200 buffer with 0.1mM PMSF containing 0.2 mg/ml Proteinase K and 2% Triton X-100, respectively. The samples were incubated for 20 min on ice. 3mM PMSF was added to stop the enzymatic activity. The cytosolic and organellar fraction was separated by ultra-centrifugation for 30 min at 100,000 × g at 4°C in a Optima™ TLX Ultracentrifuge (Beckman) using a TLA 110 rotor. The cytosolic supernatant was transferred to new tube and the organellar pellet was immediately resuspended in 60 µl SDS-sample buffer. The supernatant was treated with 1/10 volume of 110% TCA and spun down at 20,000 × g for 10 min. After discarding the supernatant, the pellet was washed once with ice cold acetone and resuspended in 60 µl SDS-sample buffer. The samples were incubated for 15 min at 37°C with slight agitation, then heated at 95°C for 5 min. The samples were cleared by centrifugation at 20,000 × g at RT before being applied to SDS-PAGE.

2.2.4.10 Blue Native Electrophoresis

Blue Native PAGE (BN) was carried out as described by Schägger and von Jagow, (1991) with modifications described in 2.2.4.10.1 and 2.2.4.10.2. If not stated, all the gel units (BN-PAGE and 2-Dimentianal-SDS-PAGE) were connected to a cooling circulator or embedded in an icebox and run in the cold room (8-9°C).

2.2.4.10.1 Preparation of the BN-Gels (gradient 4%-12%)

All solution were stored at 4°C. 49.5% of Acrylamide/Bis-acrylamide mixture (AB-mix) (49.5% T, 3% C) was made manually and filtered once by a 0.2µm filter under the vacuum. 3 × Gel buffer (pH7.0) contains 150mM BisTris and 200mM EACA. Cathode buffer B (pH7.0) contains 15mM BisTris and 50mM Tricine. Cathode buffer A is the same as Cathode buffer B with presence of 0.02% Serva Blue G250. Anode buffer (pH 7.0) contains 50mM BisTris. One protocol for one mini-Gel with 0.75mm spacer is listed as following. 2ml of each 4% and 12% separation gel solution was poured into Gradient Former (Bio-Rad). And the stacking gels should be poured before the separating gel was polymerised. The gels have to be made one day before use and stored at 4°C.

2.2.4.10.2 Preparation of the samples and Running of the BN-gels

All detergents stocked solution mentioned were made with solubilization buffer B1 (see 2.2.4.7). The aliquots of crude microsomal membrane (see 2.2.4.7) in buffer B2 were thawed on the ice and then a corresponding volume of B1 buffer containing different detergents was added to the microsomal membrane suspension to a final concentration of 12.5% glycerol and 1% Digitonin. For solubilization with other detergents, the final concentration is indicated in 2.2.4.7. After solubilization of the membrane proteins (see 2.2.4.7), the supernatant was combined with 1:10 volume of BN sample buffer (100mM BisTris (pH 7.0), 500mM EACA, 5% (w/v) Serva blue G) and applied to 0.75mm/1.5mm-thick 4-12% BN gels in a Hoefer Mighty mini vertical electrophoresis unit or a Hoefer Mighty midi vertical electrophoresis unit. Electrophoresis was run for a total 3-4 hr at 5mA in stacking Gel and 10mA in Separating Gel at 4°C. The cathode buffer A was exchanged with buffer B after the top 1/3-1/2 of the gel was covered with dye. The high molecular weight markers are Thyroglobulin (669kDa), Ferritin (443kDa), Catalase (230kDa), Lactate dehydrogenase (140kDa) and Albumin (66kDa).

2.2.4.10.3 Further procedures

2.2.4.10.3.1 Western Blotting

BN gels were equilibrated in colourless cathode buffer for 15min at RT to remove as much as possible excess Serva blue and soaked in Blotting buffer (20mM Tris-HCl, 150mM Glycine, 0.02% (w/v) SDS) containing no methanol for 10min at RT. PVDF membrane was activated with methanol completely and soaked in Blotting buffer with 7 pieces of filter paper. Protein transfer was performed for 1.5-2hrs at 100mA/ per mini-gel at cold room by using a semi dry transfer system (Hoefer Pharmacia Biotech, San Francisco, CA). From Cathode side,

the order of the sandwiches was 3 pieces of filter paper, BN gel, PVDF and final 4 pieces of filter paper (because of Coomassie dye). After blotting, the PVDF membrane was shortly destained with 100% methanol for a few seconds to remove the Coomassie dye as much as possible. Before the normal Western procedure was performed, with the help of Ponseau S. staining, HMG protein marker on the membrane was visibly marked with a ball pen. The PVDF membrane was normally immunodecorated with first antibody overnight and was able to be reprobed with different antibodies for a few times (see 2.2.4.3). Immunoblots were developed by Lumi-Light Western substrate Kit (Böhringer Mannheim).

2.2.4.10.3.2 Denaturing second dimensional SDS-PAGE

After the first dimensional BN electrophoresis (0.75mm thick, Bio-Rad mini vertical electrophoresis unit), the required BN-gel slices were excised and soaked for 15-30min in denaturing buffer (1M Tris-HCl, pH 6.8, 1%SDS and 2.5% β-ME). The gel slices were then washed for 5min for three times in the same buffer but in the absence of β-ME. Remarkably, removal of β-ME is necessary as it prevents subsequent polymerisation of an acrylamide stacking gel when the second dimension is done. The gel strip were then put horizontally on the top of a prepared 1mm thick SDS-PAGE in a mini (0.5cm high stacking gel, 4.5 cm high separating gel). The stacking gel was poured around gel slices and no bubbles should be visible between gel slices and stacking gel and one cut comb containing 1-3 wells was put on one side of gel strip. Before electrophoresis was started, the gel slices were sealed with 1% agarose. 5µl of Urea sample buffer were dropped into the cathode buffer as an indicator. Migration of proteins occurs under 20mA per gel and 150V as limit following Western detection (see 2.2.4.3)or Silver staining (see 2.2.4.2).

2.2.4.10.3.3 Endo-H in gel digestion

After the first dimensional BN electrophoresis (1.5mm-thick mini gel was used in this case) was finished, proteins were visualized with Coomassie staining (0.005% Serva Blue G-250, 50% Methanol, 7% acetate acid) for 20min followed by partial destaining for 30-60min with a solution containing 30% methanol and 7% acetate acid to remove background staining. The excised gel pieces (approximately 5mm $\times$ 1mm$\times$ 1.5mm) were cut into smaller fragments, weighed and approximately equally divided into two aliquots. The gel pieces were rinsed twice with dH_2O. Then the gel pieces were soaped in 3-4 gel volumes 100% Acetonitrile (HPLC grade) for 10-15min with occasionally short vortex. After the liquid was removed by centrifugation at 5000 $\times$ g for 2min at RT, the gel particles were dried with a Speed vacuum for 15min. The gel particles were swelled in 2 gel volume of 1 $\times$ Glycoprotein denaturing

buffer (5% (w/v) SDS, 10% (v/v) β-ME) and incubated for 30min at 56°C. After washing twice with Buffer (1% (w/v) SDS, 2% (v/v) β-ME) and water for two times, the gel particles were treated again with 100% Acetonitrile following a desiccation by Speed vacuum. The gel particles were then swelled in two gel volume of potassium phosphate washing buffer containing 0.7% (v/v) *β*-ME. 3µl Endoglycosidase H was added into one of the aliquots and incubated for 3hr at 37°C, then 1µl more Endoglycosidase H was added and incubated overnight until a total digestion 12-15 hrs at 37°C. The digestion was stopped by adding 1/6 volume of Urea sample buffer containing 1.5% (v/v) *β*-ME was added into aliquots and heated at 95°C for 5min. All the materials including liquid and gel slices were loaded into the wells on a normal SDS-PAGE. The wells was sealed with 1% agarose before the electrophoresis started.

2.2.4.10.3.4 MALDI-TOF-MS

MALDI-TOF analyses was performed in cooperation with Prof. Dr. Albert Sickmann (Robert Virchow Centre, University of Würzburg). Gels should be treated under dust-free conditions using deionized water, solvents and reagents of electrophoresis grade. Gels are exposed for 0.1% Coomassie Brilliant Blue G-250 in the mixture containing methanol/acetic acid/water (40:10:50) for 1.5 hrs and then washed with 15% acetic acid to remove unbound dye. Protein bands of interest were cut from the gel (pieces 1 x 1 mm) using clean scalpels. The samples were sent to Proteomics Department in Würzburg per normal post.

2.2.4.11 Native Co-immunoprecipitation

The native co-immunoprecipitation was carried out as following. 500OD_{600} units of Logarithmic phase yeast cells (2-3 OD_{600} units/ml) were harvested by centrifugation and washed once with 15mM ice-cold NaN_3 (15ml/100OD600 of cells). After crude microsomal membrane was isolated as mentioned in 2.2.4.7, membrane suspensions were 4-5 times diluted with solubilization buffer B1, and solubilized by adding Digitonin to a final concentration of 1%. When it was required, 5mM ATP or a 1 × ATP regenerating system (ARS) (5mM ATP, 30mM Creatine phosphate, 50µg/ml Creatine phosphate Kinase, 5mM $MgCl_2$) was added in B1 dependent on purpose. Remarkably, 5% Digitonin, 100mM ATP, 10 × ARS stock solution were made in solubilization B1 to keep consistent salt concentration. Solubilization reaction was mixed gently for one hour by using a Top-to-End rotator in the cold room. After insoluble material was removed by ultra centrifugation for 30min at 75000rpm (200,000 × g) at 4°C in a Beckman TLA 120.2 (Beckman Instruments, Palo Alto, CA), 50µl of the supernatant was taken for evaluation of total lysate protein and added to 50µl

of 2 × Urea sample buffer containing 3% (v/v) β-ME. The remaining lysate was adjusted with solubilization buffer B1 containing 1% Digitonin into five aliquots with an approximately volume 450µl/100OD/per tube. 5µl of polyclonal rabbit anti-Cdc48 antisera (a kind gift from F. Madeo), 5µl of polyclonal rabbit anti-CPY antisera, 2.5µl of affinity purified rabbit anti-Cdc48 (a kind gift from T.Sommer), 2.5µl of affinity purified rabbit anti-Sec61 (a kind gift from T.Sommer), 2.5µl monoclonal mouse anti-Myc (Calbiochem) and 2.5µl monoclonal mouse anti-HA (Babco) was added to each tube to immunoprecipitate corresponding targeted proteins, respectively. In addition, for negative control, no antibody was added. The mixture was wheeled gently for 12-16 hrs by using a Top-to-End rotator in cold room. 50µl Protein G Plus/Protein A Agarose (Calbiochem) were added to the mixture, which was then kept on wheeling for 4-5 hrs on rotator in cold room. Protein G Plus/Protein A Agarose beads were harvested by centrifugation at 1500 × g for 1min at 4°C and washed for 3-4 times with solubilization buffer B1 containing 0.5% Digitonin. Proteins were removed from the beads by addition of 80µl 1 × Urea sample buffer containing 1.5% (v/v) β-ME. All samples were incubated at 65°C for 25min following a centrifugation for 5min at 20,000 × g before 10-15 µl of samples were applied to a 9% or 5-16% SDS-PAGE.

2.2.4.12 In situ assay for chymotrypsin-like activity of the proteasome

Overlay assay was performed as following. The cells, which were freshly patched and incubated on the YPD plate at 30°C overnight, were replicated onto a sterile filter paper on a fresh YPD plate. The replica plate with the cells on the filter paper was incubated at 30°C for 24 hrs. After being transferred into a glass Petri dish, the cells on the filter paper were permeabilized by 10ml chloroform for 15min. The dried filter paper was then transferred to a plastic Petri dish and overlayed carefully with 10ml substrate solution (1% agar, 9.7ml 50mM Tris-HCl (pH 8.0), 300µl of 10mM N-Cbz-Gly-Gly-Leu-pNA in DMSO), any bubble on the edge of filter paper must be seriously removed. After being incubated at 37°C for 3hrs, the overlaid filter paper was incubated at RT for 5min with 10ml buffer 1 (0.1% (w/v) sodium nitrite in 1M HCl) and 5min with 10ml buffer 2 (0.5% (w/v) ammonium sulphamate in 1M HCl) respectively. After addition of 10ml of buffer 3 (0.05% (w/v) 1-naphthyl-ethylendiamine in 47% (v/v) Ethanol), the overlaid filter paper was incubated at RT until positive colonies were visualized in pink colour. To stop the colour reaction, the overlaid filter paper was rinsed with water immediately for three times.

To quantify the chymotrypsin-like activity, 1 OD_{600} of logarithmic phase cells (OD_{600}1.0 units/ml) were washed with water and the moist cell pellet were permeabilized by 0.8ml Chloroform for 15min. After addition of 105µl of dH_2O, 100µl of the upper aqueous

protein fraction was transferred into a new microtube containing 100µl reaction buffer (0.1M Tris-HCl (pH 8.0), 0.15mM succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin) and incubated for 10min at 37°C with a gentle agitation. The reaction was stopped by adding of 1ml 100% of ice-cold ethanol. After a clarity by centrifugation at 20,000 × g for 3min, the fluorescence was measured at 460nm, using an excitation wavelength of 380nm.

3. Results

3.1 New Substrates constructed for trapping the retrotranslocon of the ER

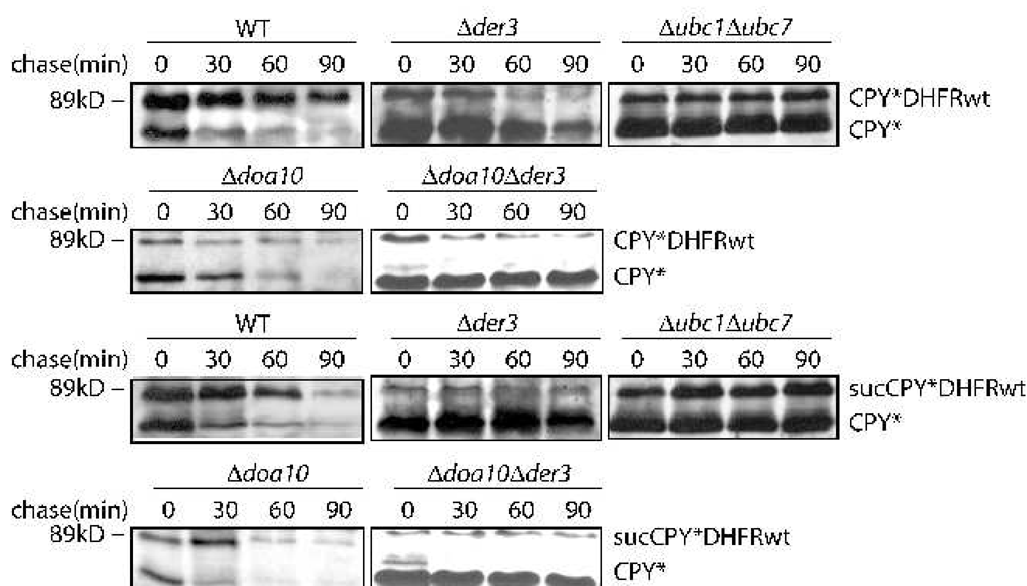
In order to investigate whether the Sec61 translocon, the ER protein import channel, may also be involved in the process of retrograde transport of malfolded proteins to the cytosol (export), a series of CPY* fusion protein substrates with special structural characteristics were engineered. As Sec61p is part of the protein import channel, the rationale behind the experimental design is that demonstration of a retrograde protein transport channel with Sec61p as a component would be possible if channel and substrate could be trapped during export. Provided that one of these fusion protein substrates would jam the export channel, it would be possible to determine the composition of the retrotranslocon. In addition, new information on ERAD as a whole could be obtained by following the retrotranslocation of such fusion proteins from the ER back to the cytosol.

3.1.1 Characteristics of CPY fusion proteins*

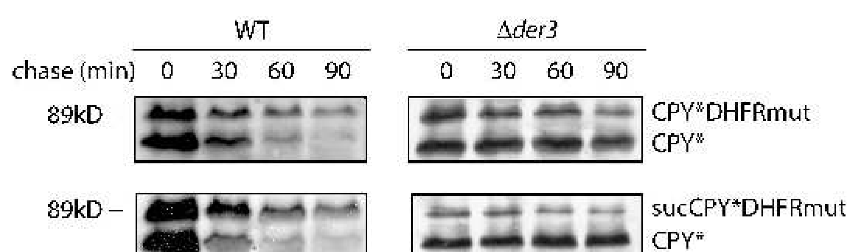
CPY*, a model substrate in the study of ERAD, is a mutant version of the soluble vacuolar protease carboxypeptidase Y (yscY), encoded by the *PRC1* gene (Finger et al., 1993; Wolf and Schäfer, 2005). Different CPY* fusion proteins containing tightly folded domains were previously constructed (Hitt and Wolf, unpublished data) and are further analysed as ERAD substrates in this work. One of the CPY* fusion partners is the tightly folded cytosolic enzyme DHFR from mouse (Dihydrofolate Reductase) (Eilers and Schatz, 1986; Schlenstedt et al., 1994; Mingarro et al., 2000). The DHFR domain of a CPY*-DHFRwt fusion was believed to be still able to be folded completely in the ER. CPY*-DHFRmut is a mutant form of DHFR which can no longer fold properly. CPY*-DHFRmut was used as a malfolded control for CPY*-DHFRwt (Thillet et al, 1998). CPY* was also fused to Rhodanese and BPTI (Bovine Pancreatic Trypsin Inhibitor), both of which are small and compact proteins difficult to unfold (Ploegman et al., 1978; Miller et al., 1991; Parekh et al., 1995; Kowalski et al., 1998a,b). CPY*DHFR, CPY*BPTI-HA_3 and CPY*Rhodanese were cloned behind the promoter and signal sequence of *PRC1*. I wanted to test whether ERAD and, more specifically, retrotranslocation, is affected by tightly folded domains located in the ER due to fusion to CPY*. Another CPY* fusion was created using invertase. Invertase, the product of the *SUC2* gene, is a large (60 kDa) and heavily glycosylated secreted yeast protein (Kern et al., 1992; Gilstring and Ljungdahl, 2000). CPY*Suc2p was cloned behind the promoter and signal sequence of *PRC1*. This fusion protein was constructed to determine the flexibility of

the retrotranslocation channel and to see whether a malfolded and heavily glycosylated protein could be retrotranslocated back into cytosol. CPY* is post-translationally translocated into the ER. To investigate whether there are differences in the degradation of malfolded proteins co- and post-translationally translocated into the ER, the *CPY* signal sequence on CPY*DHFRwt and CPY*DHFRmut was replaced with the signal sequence of Suc2p, a co-translationally translocated protein. These constructs were designated as sucCPY*DHFRwt and sucCPY*DHFRmut. In addition, Suc2CPY* was constructed, in which Suc2p is N-terminally fused to CPY*, using the *Suc2p* promoter and signal sequence. We hoped that these CPY* fusion proteins, which are expected to behave as *bona fide* ERAD substrates due to the presence of the CPY* moiety, may block the retrotranslocon and halt export out of the ER due to the presence of tightly folded domains and/or a large number of glycan chains.

A



B



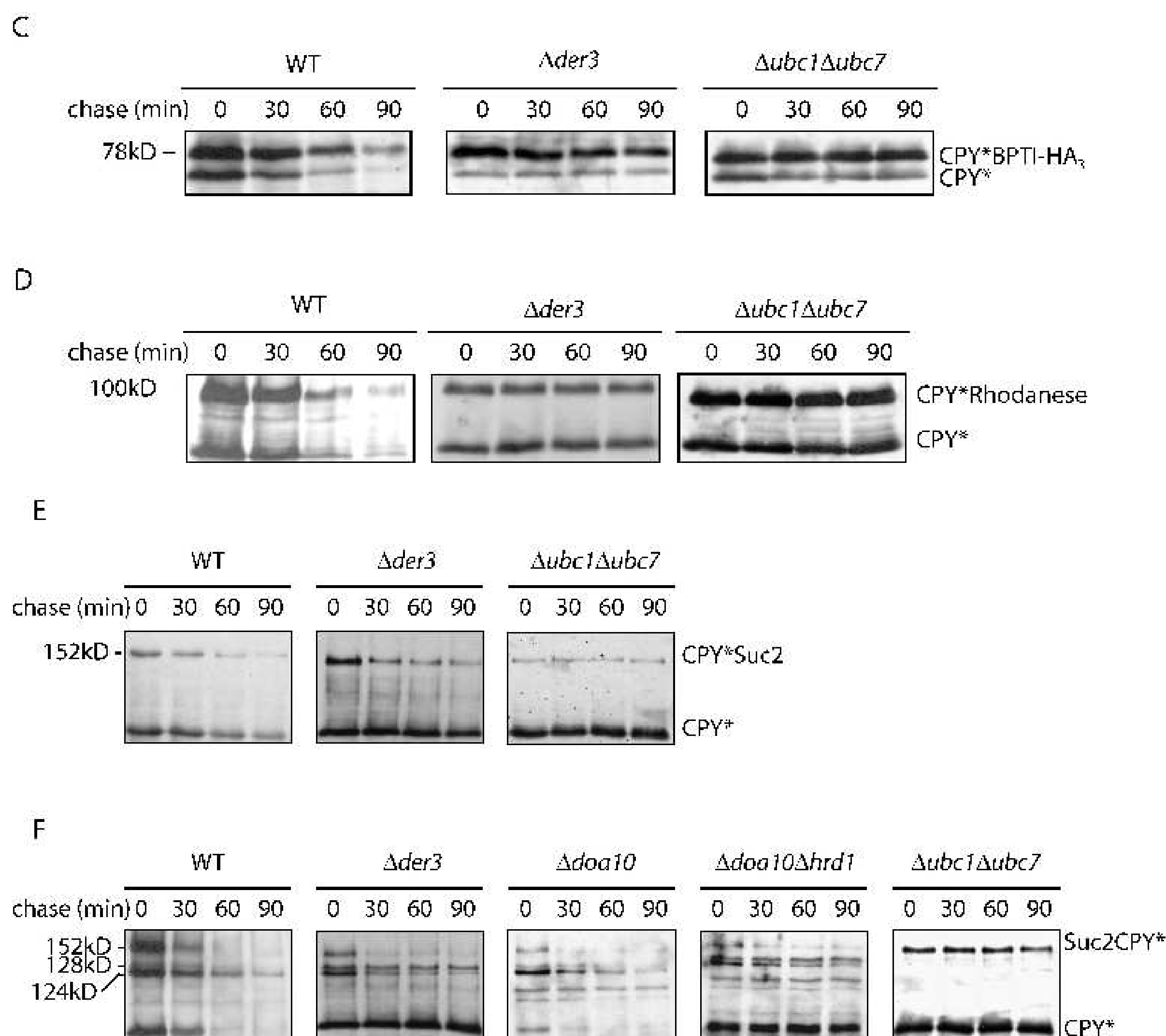


Figure 5. ER-degradation of the misfolded CPY* fusion proteins. Cycloheximide chase experiments performed in wild type (W303-1C) and the ERAD defective strains Δ*der3*, Δ*doa10*, Δ*doa10*Δ*hrd1* and Δ*ubc1*Δ*ubc7* expressing both CPY* and the CPY* fusion proteins CPY*DHFRwt and sucCPY*DHFRwt (A), CPY*DHFRmut and sucCPY*DHFRmut (B), CPY*BPTI-HA$_3$ (C), CPY*Rhodanese (D), CPY*SUC2 (E) and SUC2CPY* (F), respectively. Proteins were visualized using CPY antibodies.

3.1.2 Degradation of CPY fusion proteins requires components of the ERAD system*

In previous studies, several components of the ER degradation system were described to be crucial for the efficient degradation of CPY*. These include the E2 ubiquitin conjugating enzymes Ubc1p and Ubc7p, and the E3 ubiquitin ligase Der3p (reviewed in the Introduction). To ascertain that the degradation of the various CPY*-fusion proteins generated depends on the same components, the fate of CPY* fusion proteins was analysed in the following ERAD deficient yeast strains: *Δubc1Δubc7*, *Δder3*, and *Δdoa10*, lacking the second

ERAD E3 known to date (Fig. 5). The stability of CPY* and CPY* fusion proteins was analysed by Western blotting after inhibition of proteins synthesis by cycloheximide. The approximate molecular mass of CPY*, CPY*DHFR (wt or mut) and sucCPY*DHFR (wt or mut), CPY*BPTI, CPY*Rhodanese, CPY*Suc2 is 67kDa, 89 kDa, 78 kDa, 100 kDa and 152 kDa, respectively (Fig. 5). The degradation of CPY*DHFRwt, sucCPY*DHFRwt, CPY*DHFRmut, sucCPY*DHFRmut and CPY*BPTI is slower than the turnover of CPY* in wild type cells at 30°C (Fig. 5A and B). CPY*Rhodanese, on the other hand, is degraded with similar kinetics to CPY* in wild type cells at 30°C (Fig. 5C, D, E and F). The DHFR, BPTI and Rhodanese fusion proteins are completely stable for the extent of the chase period in cells devoid of the ubiquitin conjugating enzymes Ubc1p and Ubc7p (*Δubc1Δubc7*) (Fig. 5). CPY* and the fusions CPY*Rhodanese, CPY*BPTI, CPY*DHFRmut, sucCPY*DHFRmut were also stabilized in *Δder3* cells (Fig. 5B, C and D). However, the Der3p dependence of CPY*DHFRwt and sucCPY*DHFRwt is less clear. This is surprising because Der3p/Hrd1p functions as an ER-membrane E3 ligase together with the E2´s Ubc1p and Ubc7p, and all three are essential components of CPY* degradation. Recently, Doa10p was identified as a second ER-membrane localized E3 and it has been involved in the degradation of Ubc6p and Ste6p (Swanson et al., 2001; Huyer et al., 2004). Ubc6p and Ste6p are degraded independent of Der3p or Sec61p (Swanson et al., 2001; Huyer et al., 2004). In order to investigate if Doa10p plays a role in the turnover of CPY*DHFRwt and sucCPY*DHFRwt, these proteins were expressed in *Δdoa10* and *Δdoa10Δder3* cells. As shown in Fig. 5A, sucCPY*DHFRwt was stabilized only when both, Doa10p and Der3p, are missing. The turnover of CPY*DHFRwt in *Δdoa10Δder3* cells appears similar to its turnover in wild type and *Δdoa10* cells. This data indicates that, as suspected, the two E3´s, Doa10p and Der3p/Hrd1p, may have overlapping target substrates to act upon.

CPY* fused to invertase exhibits a marked difference in migration depending on whether Suc2p is in the N- (Suc2CPY*) or C- (CPY*Suc2) terminus. CPY*Suc2p migrates at a position expected for a protein of 152 kDa (calculated). CPY*Suc2p is degraded in WT and *Δder3* cells, and stabilized in the *Δubc1Δubc7* double mutant. Suc2CPY*, on the other hand, migrates as three species: the full-length protein of 152 kDa and two smaller species of 128 kDa and 124 kDa. Whereas the full-length (152 kDa) protein appears to be degraded as quickly as CPY* either in WT or *Δder3* and *Δdoa10* cells, the two smaller species exhibit different, partially impaired degradation kinetics (Fig. 5 E, F). Interestingly, Suc2CPY* migrates as a single 152 kDa species in the *Δubc1Δubc7* strain and it is completely stable over the duration of the chase period (Fig. 5F). The 152 kDa species of CPY*Suc2 and Suc2CPY*

corresponds to the fully glycosylated protein. We suspected that the 128 kDa and 124 kDa species of Suc2CPY* (Fig. 5F) are underglycosylated forms. Deglycosylation with endoglycosidase H (*E*ndo H) *in vitro* and block of N-glycosylation with tunicamycin *in vivo* revealed that the 152 kDa species dropped in molecular mass, indicating that these are fully glycosylated CPY*Suc2 and Suc2CPY* imported into the ER. The 128 kDa Suc2CPY* is also a glycosylated species, since its size decreases following both of treatments (Fig. 6A, B).

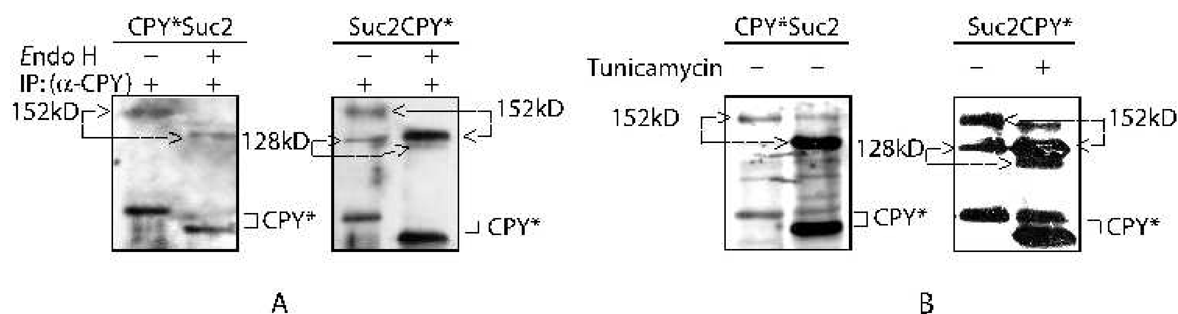


Figure 6. Glycosylation of CPY*Suc2 and Suc2CPY*. Lysates of W303-1C cells expressing CPY*Suc2 or Suc2CPY* were analysed by SDS-PAGE followed by immunoblotting with monoclonal antibodies against CPY. Prior to lysis, CPY*Suc2 or Suc2CPY* were immunoprecipitated from cell extracts with polyclonal antibodies against CPY and treated with endoglycosidase H (A) or the cells were treated with or without tunicamycin *in vivo* for 1 hour (B).

Together, this data suggest that, like CPY*, the degradation of the CPY* fusion proteins examined depends on ERAD. Although the fusion proteins contain a compactly folded or heavily glycosylated domain, they do not compromise the retrotranslocation process and are degraded via ubiquitin system. Since none of the fusion proteins disturb ERAD considerably under WT conditions, they did not seem to be suitable for use in experiments geared toward the identification of a retrotranslocation channel.

*3.1.3 A useful bait: CPY*GAr215*

Recent data suggest that ERAD substrates are polyubiquitinated and then extracted out of ER lumen or membrane in an ATP-dependent manner either via the AAA ATPase complex $Cdc48^{Ufd1\text{-}Npl4p}$ or directly via the proteasome (Ye et al., 2001; Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2003; Medicherla et al., 2004; Elkabetz et al., 2004; Richly et al., 2005; Bazirgan and Hampton, 2005). Since both fully folded and heavily glycosylated ER proteins appear to be degraded via ERAD (see sections 3.1.1 and 3.1.2), another way to block the retrotranslocon could be by disturbing the pulling machinery of ERAD. Defective

membrane extraction due to disturbance of the Cdc48$^{Ufd1-Npl4p}$ complex or of the 26S proteasome may lead to obstruction of the retrotranslocation channel, thereby allowing its detection. It has been shown that the EBNA virus has evolved a mechanism to escape immune detection via a long stretch of Gly-Ala repeats (GAr) in the EBNA1 protein that blocks its proteasomal proteolysis (Levitskaya et al., 1995; Levitskaya et al., 1997; Heessen et al., 2003). Fusions of Gly-Ala repeats to a number of proteasome substrates decrease their turnover by the proteasome (Dantuma et al., 2000; Heessen et al., 2002). Recently it was shown that Gly-Ala repeats introduced into the proteasomal substrate ornithine decarboxylase (ODC), stall the degradation of this enzyme (Hoyt et al., 2003, Zhang and Coffino, 2004). Interestingly, a degradation intermediate of ODC was observed whose length is compatible with a sequence loss up to the Gly-Ala repeats. It was shown that this is most likely due to an "idling" of the 19S cap ATPases of the proteasome unable to "grab" the unstructured, "slippery" Gly-Ala repeats (Zhang and Coffino, 2004). Independently, we also decided to engineer CPY* constructs containing Gly-Ala repeats (Fig. 7) to test whether transport of this substrate out of the ER would block the retrotranslocation channel.

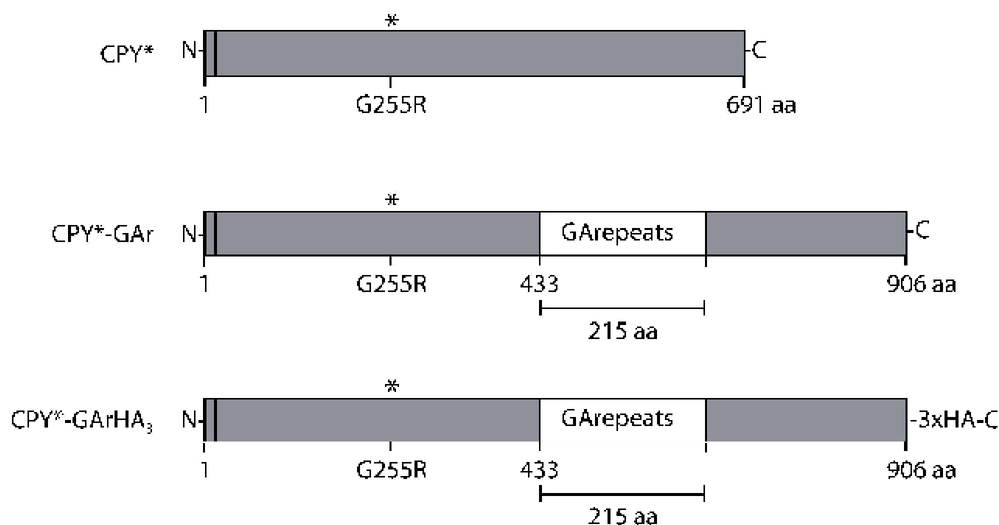


Figure 7. Schematic representation of CPY* and Gly-Ala repeat fusion constructs of CPY*, CPY*GAr215 and CPY*GAr215HA$_3$. To construct CPY*GAr215, the glycine-alanine (GA) repeats (215 amino acids) were introduced into CPY* at amino acid position 433.

The CPY*GAr215 and CPY*-GAr215HA$_3$ hybrids were constructed by C. Alberola and Z. Kostova (C. Alberola (2003), Diplomarbeit, Universität Stuttgart) and shown to undergo ER-associated degradation. Cycloheximide chase experiments show that CPY*GAr215 (~98 kDa) and CPY*-GAr215HA$_3$ (~100 kDa) are degraded within 90 min in

wild type cells deleted for CPY (Fig. 8A) with kinetics similar to CPY*. Interestingly, a ~78 kDa intermediate of CPY*-GAr215HA$_3$ appears and accumulates during these chases (C. Alberola (2003), Diplomarbeit, Universität Stuttgart; T. Bohnacker (2004), Diplomarbeit, Universität Stuttgart) (Fig. 8A). This intermediate can no longer be detected when the same blots are reprobed with the anti-HA antibody, indicating that the C-terminal HA$_3$ epitope has been cleaved off. The ~22 kDa reduction in molecular mass together with loss of the C-terminal HA$_3$-tag indicates that the C-terminus has been removed up to the Gly-Ala repeats and suggest that degradation commences at the C-terminus of the protein. Furthermore, in Δ*der3* and Δ*ubc1*Δ*ubc7* cells defective in the ERAD ubiquitination machinery, and in *pre1-1* and *cim3-1* cells defective in proteasomal proteolysis, only full length CPY*-GAr215HA$_3$ accumulates during a 90min chase (Fig. 8B), reinforcing the idea that the 78 kDa species is a degradation intermediate of CPY*-GAr215HA$_3$.

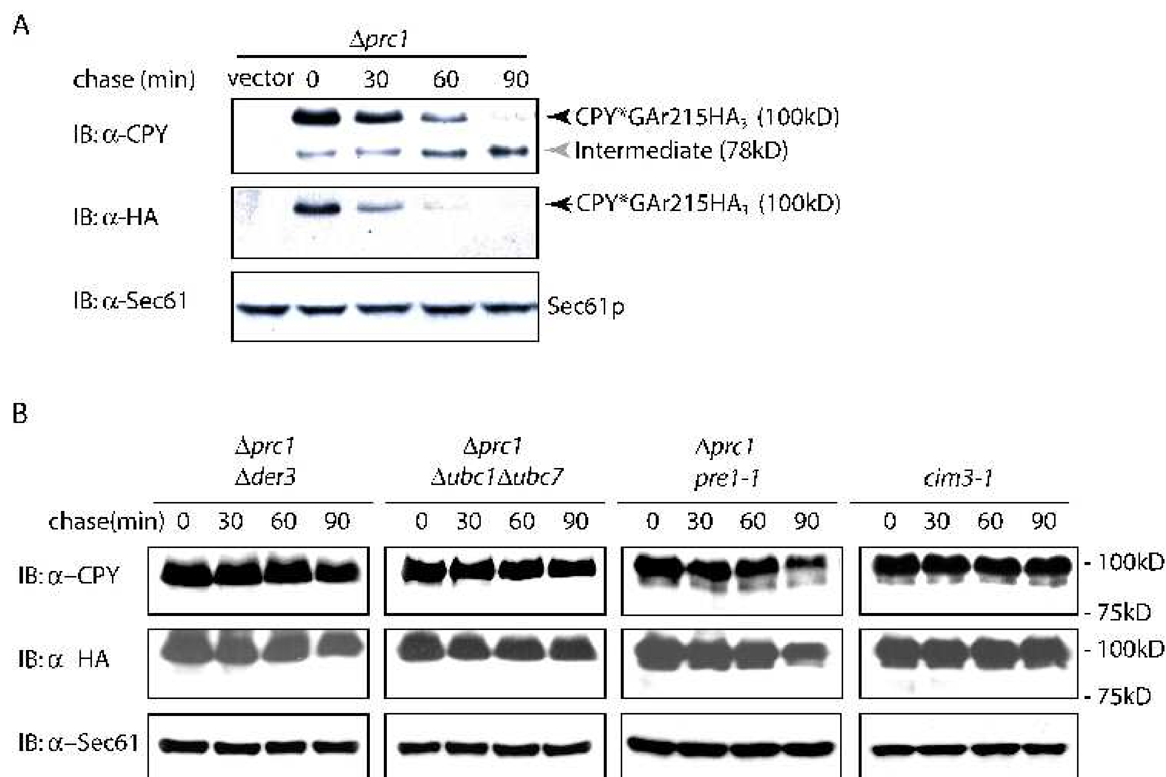


Figure 8. Degradation of CPY*GAr215HA$_3$ depends on ERAD ubiquitination machinery and the proteasome. Cycloheximide chase experiments were performed in wild type cells W303-1B lacking CPY (Δ*prc1*), ERAD mutant strains Δ*der3*, Δ*ubc1*Δ*ubc7* and proteasomal mutants *pre1-1* and *cim3-1* expressing pRS316-CPY*GAr215HA$_3$. Whole cell extracts were analysed by SDS-PAGE and immunoblotting with antibodies against CPY and HA, respectively. Sec61p levels at each time point were determined as loading controls.

To prove that the 78 kDa species does not originate from a translational defect and inefficient import into the ER caused by the Gly-Ala repeats, a plasmid was constructed by replacing *CPY* signal sequence on CPY*GAr215HA$_3$ with the *SUC2* signal sequence. The *SUC2* signal sequence should induce co-translational transport of CPY*GAr215HA$_3$ into the ER (see material and methods). Upon expression in W303ΔC cells, both the full length (~100 kDa) and intermediate (~78 kDa) forms of co-translationally imported CPY*GAr215HA$_3$ were visible (Fig. 9A), indicating that this 78 kDa immune-signal was indeed a processing product during retrotranslocation of the CPY*GAr215HA$_3$ protein. To show that the full length CPY*GAr215HA$_3$ has entered the ER and that the 78 kDa intermediate was generated after ER import, the extent of N-glycosylation of both forms of the CPY*GAr215HA$_3$ was determined. The shift in molecular mass following *in vitro E*ndo H treatment shows that both the full length and intermediate forms of co- and post-translationally imported CPY*GAr215HA$_3$ are N-glycosylated (Fig. 9, A and B).

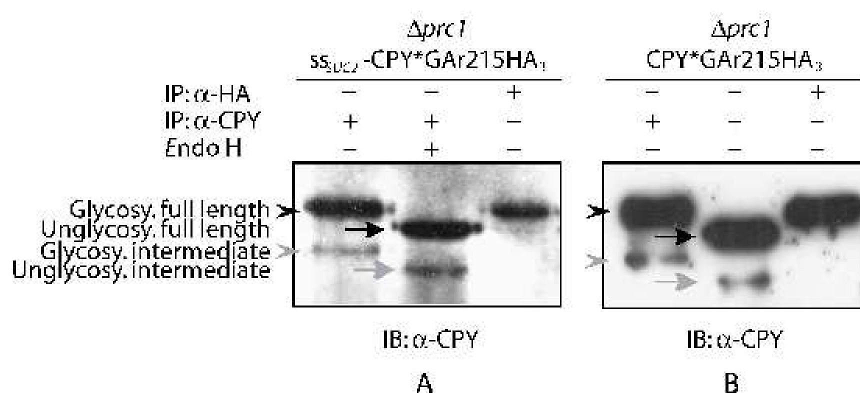


Figure 9. Both full length and the 78 kDa intermediate of CPY*GAr215HA$_3$ are glycosylated. ss$_{SUC2}$-CPY*GAr215HA$_3$ (A) and CPY*GAr215HA$_3$ (B) were immunoprecipitated with antibodies against HA and CPY from W303ΔC (Δ*prc1*) cell extracts expressing ss$_{SUC2}$-CPY*GAr215HA$_3$ from plasmid pXL3163 (A) or CPY*GAr215HA$_3$ from plasmid pZK129 (B) and treated with *E*ndo H before analysis by SDS-PAGE and immunoblotting with monoclonal antibodies against CPY.

To demonstrate that full length CPY*GAr215HA$_3$ and its 78 kDa intermediate fully reside within the ER, a protease protection experiment was performed. Cell fractionation and proteinase K treatment of pelleted microsomes (P) and supernatant (S) fractions demonstrate that both forms are present in the microsomal fraction and are fully protected from proteinase K digestion (Fig. 10). Taken together, these results show that CPY*GAr215HA$_3$ is an ERAD substrate which is not fully degraded but processed to a 78 kDa intermediate which resides in the ER. The appearance of the intermediate in the ER but not in the cytosol suggests that the full length CPY*GAr215HA$_3$ does not completely leave the ER. Instead, its retrotranslocation,

which obviously begins at the C-terminal end of the molecule, seems to be interrupted when it reaches the Gly-Ala repeats. The Gly-Ala repeats within CPY* may mimic the "idling" at the proteasome observed with ODC by Zhang and Coffino (2004). In this case, however, the idling occurs at the retrotranslocation step and the partially degraded protein may slip back into the ER or it may remain associated with the retrotranslocon. If the 78kDa intermediate remains engaged with the retrotranslocation channel, this would prevent the disassembly of the channel and allow for its biochemical isolation.

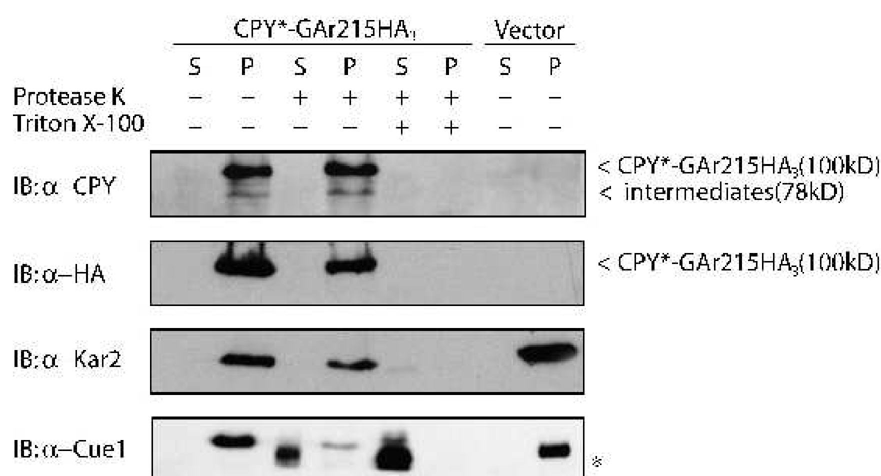


Figure 10. Both full length and the 78 kDa intermediate of CPY*GAr215HA$_3$ are located in the ER. Proteinase K protection assays were performed according to 2.2.4.9 in W303ΔC cells expressing CPY*GAr215HA$_3$ from plasmid pZK129. After protease deactivation, supernatant and pellet fractions were separated and then analysed by SDS-PAGE and immunoblotting with anti-CPY and anti-HA antibodies. For control of vesicle integrity and protease activity, blots were probed with anti-Cue1p (ER transmembrane protein) and anti-Kar2p (ER lumenal protein) antibodies. The asterisk (*) denotes the cytosolic fragment originating from the incomplete digestion of Cue1p.

Previous genetic experiments suggest that the Sec61 ER-protein import channel protein may also function as the retrograde channel for export of malfolded proteins out of the ER (Plemper et al., 1997; Plemper et al., 1999). The ER-import channel is an extremely dynamic entity, which disassembles following protein translocation (Dashaies et al., 1991; Rapoport et al., 1992). It is very likely that the ER-export channel would also behave similarly, thereby ensuring Sec61p's availability for the assembly of import or export channels according to the cell's needs. It was reasoned that if the 78 kDa intermediate of CPY*GAr215HA$_3$ can not disengage from a Sec61p containing export channel, the level of Sec61p necessary for protein import would diminish therefore interfering with efficient cell growth. To establish this, the temperature sensitive growth defect of Sec61-2 cells was

utilized, in which the level of Sec61-2p is reduced due to degradation of the mutant protein (Biederer et al., 1996). It was observed that, while wild type cells expressing malfolded CPY*HA_3 grow like cells containing an empty vector, there is a slight but reproducible growth reduction in *sec61-2* cells expressing CPY*GAr215 at 35°C (Fig. 11B). A similar growth defect is observed when CPY*GAr215 is expressed in *pre1-1* cells defective in proteasome function (Fig. 11C). One possible explanation could be that the "slippery" CPY*GAr215 cannot be degraded or efficiently processed by the proteasome (see section 3.1.3, Fig. 8) and remains longer associated with both the proteasome and a Sec61p containing retrotranslocation channel. Based on this theory, we decided to undertake the biochemical identification of the retrograde export channel using CPY*GAr215 as substrate.

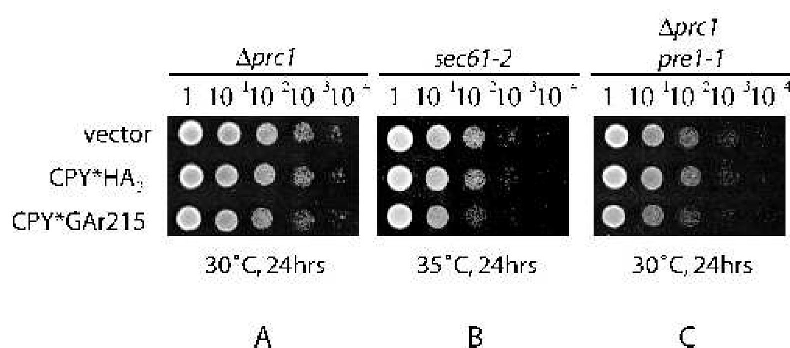


Figure 11. Full length and 78 kDa intermediate of CPY*GAr215 interfere with cell growth. Exponentially growing cells of wild type W303ΔC (Δ*prc1*) (A), cells containing an unstable Sec61 channel protein *sec61-2* (B) and mutant cells defective in proteasomal activity Δ*prc1pre1-1* (C) expressing an empty vector pRS316, CPY*HA_3 or CPY*GAr215 under *TDH3* promoter from plasmids pCT42 and pXL3164, respectively, were dropped onto CM-URA^- plates in serial tenfold dilutions and incubated for 24hrs at 30°C or 35°C.

3.2 Several native protein complexes were found by using Blue Native Electrophoresis

The discontinuous system of "Blue Native" polyacrylamide gel-electrophoresis (BN-PAGE) has been successfully employed to identify membrane protein complexes of purified mitochondria (Schägger and von Jagow, 1991). BN-PAGE is an attractive method for membrane protein research because it allows a rapid assessment of the state of protein complexes and, therefore, has the potential of the direct detection of dynamic changes occurring within a certain complex. In this method biological membranes are solubilized using mild neutral detergents like digitonin and aminocaproic acid, which retain protein complexes in native conditions. After solubilization, an anionic dye (Coomassie blue G-250), which binds to the surface of all membrane and to many water-soluble proteins, is added. Just like following addition of SDS, proteins assume a negative net charge from Coomassie blue

G-250, but are not denatured (Schägger and von Jagow, 1991, Schägger, 2001). Genetic analyses, combined with biochemical methods such as co-immunoprecipitation and crosslinking, have identified a number of proteins involved in the retrotranslocation process from the ER to the cytosol. These components are, in some cases, shown, and in others, believed to interact with the Sec61-translocon (see section 1.1). I adopted the BN-PAGE system, in conjunction to Western blot analysis, to validate known ERAD components and interactions, to identify new ERAD associated membrane proteins and complexes, and, finally, to investigate whether Sec61p is actually involved in retrotranslocation.

3.2.1 Purification and solubilization of microsomal membranes

The first step was to establish conditions for optimal cell lysis and solubilization of Sec61 containing microsomal membranes. Following multiple trials, I concluded that the spheroplasting procedure using Oxalyticase digestion was the most efficient and reliable method for yeast cell lysis (see methods). The capacity of non-ionic detergents to solubilize most integral membrane proteins also depends on the ionic strength of the solubilization buffer. Previous data has shown that a high concentration of K^+ ions is a prerequisite for the isolation of functional Sec61 translocon complex. However, high concentration of salts is incompatible with the principle of BN and especially K^+ ions are very harmful. Schägger and von Jagow, 1991, suggest the addition of 500mM 6-aminocaproic acid in the solubilization buffer. I established that 500mM 6-aminocaproic acid can replace 500mM KOAc from the electrophoresis system without disturbing the efficiency of solubilization, especially for the Sec61 complex (data not shown). At least 200mM sorbitol or 10% glycerol was added in all buffers to isolate and store active protein complexes (data not shown).

Sec61p contains ten highly hydrophobic transmembrane domains. I experimented with several mild and non-ionic detergents such as Triton X-100, Deoxy-BigCHAP (DBC), *n*-Dodecyl-β-D-maltoside (DDM), and digitonin to determine the best detergent for solubilization of intact Sec61 complexes. I observed that stability of Sec61p gradually decreased using digitonin, DBC, DDM and Triton X-100, respectively. However, efficient solubilization of the Sec61 complex was best in Triton X-100, followed by digitonin, DBC, and DDM (data not shown). The ERAD components Der3p and Hrd3p were found in a stable >700 kDa complex independent of the detergent used (digitonin, DBC or DDM). Triton X-100 proved to be too harsh, yielding a complex pattern of immunoreactive bands that varied with the detergent concentration (data not shown). I determined that, for the purposes of this study, digitonin was the mildest detergent that generated a reproducible pattern, independent of protein concentration and ratio between protein concentration and detergent (see 3.2.2.2).

Therefore, digitonin was used in the following work to isolate ER-protein translocation complexes.

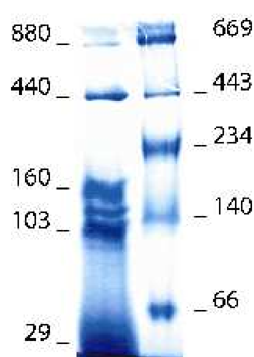


Figure 12. Protein markers for BN-PAGE. 5-13.5% BN-PAGE was prepared. On the right lane, 5 µg of standard marker proteins from Sigma were loaded (Thyroglobulin 669kD, Ferritin 443kD, Catalase 234kD, Lactate Dehydrogenase, 140kD and BSA, 66kD. Thyroglobulin (669kD) migrates close to the ferritin dimer (880kD). On the left lane, 10 µg of Apo-ferritin (443kD and 880kD) and 20 µg Carbonic-anhydrase (29 kD, 103kD, 152kD, 160kD) from Amersham were loaded.

The linear range of molecular weights on BN-PAGE proved to be between 29 kDa and 500 kDa. Above this range linearity is lost, as shown for the ferritin dimer (880 kDa), which migrates at the same position as thyroglobulin (667 kDa) (Fig. 12 left lane vs. right lane). Therefore, BN is most likely a qualitative rather than a quantitative method for judging the composition of protein complexes from molecular mass, especially when considering that migration pattern is also influenced by overall shape.

3.2.2 Blue Native electrophoresis analysis of protein complexes involved in the ER retrotranslocation pathway

To examine the organization of ER retrotranslocation pathway components, Blue Native electrophoresis was applied. Western blotting was performed with antibodies against ERAD substrates and other known components. Other researchers have found that monoclonal antibodies are not optimal for BN analysis (personal communication). It was suggested that polyclonal antibodies used for BN have to be affinity purified because Coomassie dye may disturb the identification of the proteins. Antibody against Der3p made by our lab is polyclonal, but it is not affinity purified. I performed a preliminary experiment to examine the specificity of antibodies against CPY (monoclonal) and Der3p (polyclonal) to be used in BN analysis. A centromeric plasmid pHIT341 expressing CPY* or an empty vector pRS316 was transformed into Δ*prc1* cells. The microsomal membranes from these transformants were isolated and analysed by BN. With antibody against CPY, CPY* has a smeary signal at > 700 kDa and a stronger one at ~100 kDa on BN-PAGE (Fig. 13 lane 1 vs. lane 2). It seems that CPY* is mainly involved in these two protein complexes. It is not clear why the signal of CPY* is a smear on BN-PAGE. On SDS-PAGE, the Der3p antibody unspecifically interacts with a protein at 50 kDa. To identify the real signal of Der3p on BN-

PAGE, the microsomal membranes from W303-1C and Δ*der3* cells were analysed by BN. With antibody against Der3p, BN analysis shows that Der3p has one strong signal at >700 kDa and a non-specific signal at ~430 kDa (Fig. 13 lane 5 vs. lane 6).

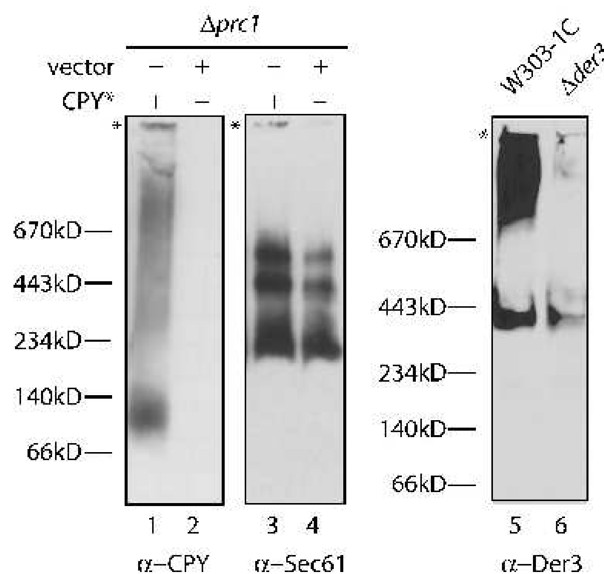


Figure 13 Specificity of antibodies against CPY and Der3p on BN-PAGE 1% digitonin solubilized microsomal membranes were analysed by 4-12% BN-PAGE. Western analysis was performed with anti-CPY (lanes 1,2) or anti-Der3 (lanes 5,6) antibodies. Sec61p levels were determined as loading controls (lanes 3,4). The asterisk (*) denotes the bottom of the wells of the BN-PAGE.

3.2.2.1 Der3p and Hrd3p occurs as a complex which acts as part of the retro-translocon

Based on crosslinking experiments and genetic data, it was suggested that Der3p and Hrd3p form a sub-complex in the ER membrane, which engages in ER lumen-to-cytosol communication required to regulate Der3p stability and coordinate ERAD events on both sides of the ER membrane (Plemper et al., 1999; Gardner et al., 2000; Deak and Wolf, 2001). To examine whether this sub-complex really exists in the cells, I transformed Δ*hrd3* cells with the centromeric plasmid pYS14 expressing HA_3-Hrd3p. Microsomal membranes were prepared from these transformants and analysed by BN. A protein complex migrating above 700 kDa could be identified (Fig.14A, lanes 1, 4 black arrowhead), containing both Der3p and Hrd3p, noted as Der3p/Hrd3p complex I. Besides this, an additional Hrd3p complex around 140 kDa can be observed by using anti-HA antibody (Fig.14A, lanes 5, 6 white arrowhead).

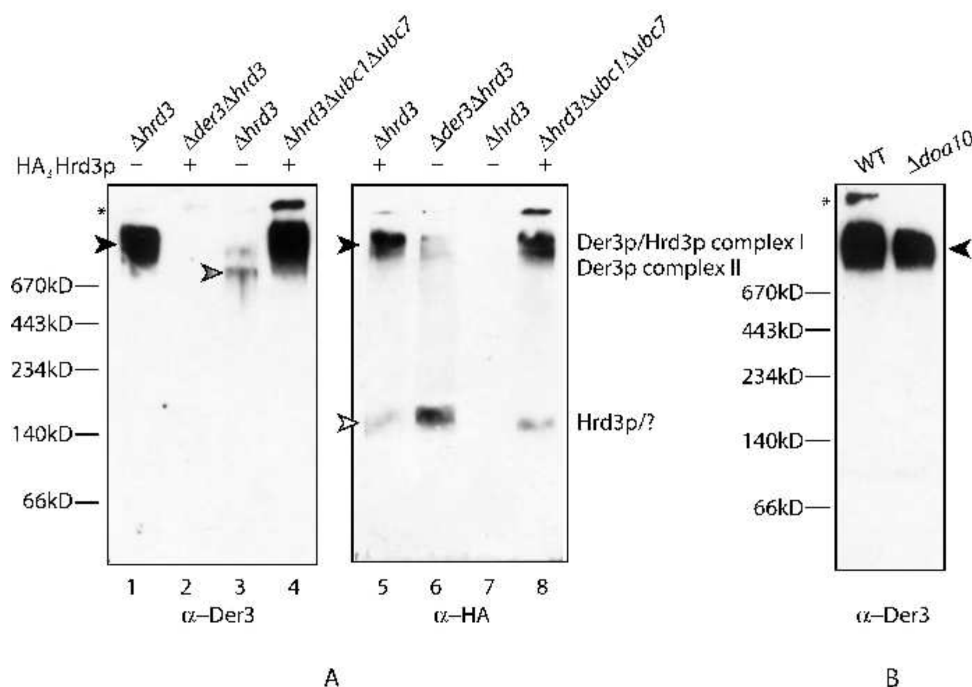


Figure 14. BN-PAGE determines the existence of high molecular mass Der3/Hrd3 protein complexes. 1% digitonin solubilized microsomal membranes were subjected to 4-12% BN-PAGE following immunoblotting with anti-Der3 or anti-HA antibodies. The asterisk (*) denotes the bottom of the wells of the BN-PAGE. A, Δ*hrd3* cells as well as Δ*hrd3*, Δ*der3*Δ*hrd3* and Δ*hrd3*Δ*ubc1*Δ*ubc7*cells expressing HA_3-Hrd3p from plasmid pYS14 were analysed by BN. Black arrowhead indicates Der3p/Hrd3p complex I. grey arrowhead indicates Der3p complex II. White arrowhead indicates 140 kDa Hrd3p containing protein complex. B, Wild type (MHY1366, *prc1-1*) and Δ*doa10* (MHY1651, *prc1-1*) cells were analysed by BN. The diagram shows two different ER membrane resident E3 complexes.

The presence of a strong Hrd3p signal in Der3p/Hrd3p complex I and a weak one in the 140 kDa complex in wild type cells (Δ*hrd3*+HA_3Hrd3p) is totally reversed in Δ*der3* cells

(Fig.14A, lane 5 vs. lane 6): deletion of *DER3* leads to destruction of the Der3/Hrd3p complex I and most Hrd3p migrates at a lower molecular mass around 140 kDa. Hrd3p is a protein with one single transmembrane domain and has a molecular mass around 95 kDa. This smaller Hrd3p containing protein complex around 140 kDa might contain other unknown protein components or it may be due to a mobility difference in the BN system. Gardner et al (2000) observed that protein levels of Der3p and Hrd3p in the cells are similar. They further proposed that stabilization of Der3p by forming a stoichiometric complex with Hrd3p might be one function of Hrd3p in ERAD mechanism. It is well known that Der3p is degraded very quickly in Δ*hrd3* cells (Plemper et al., 1999). It is proposed that lack of Hrd3p leads to destabilization of Der3p which due to self-ubiquitination undergoes proteasome proteolysis (Plemper et al., 1999; Deak and Wolf, 2001). Using anti-Der3p antibodies, BN analysis clearly shows that the Der3p containing Der3p/Hrd3p complex I disassembles in Δ*hrd3* cells (Fig. 14A, lane 1 vs. lane 3). When Der3p/Hrd3p complex I disassembles in Δ*hrd3* cells, two weaker Der3p signals can be detected, suggesting that there may be a newly formed Der3p containing protein complex of smaller amount in the cells lacking Hrd3p, noted as Der3p complex II (Fig.14A, lane 3 grey arrowhead). Most likely, this indicates that the residual amount of Der3p, which is not degraded in the absence of Hrd3p, is still bound to components of the membrane-associated ERAD machinery.

To test whether an ubiquitin conjugating enzyme E2 is part of the Der3/Hrd3 protein complex I, I transformed Δ*hrd3*Δ*ubc1*Δ*ubc7* cells with the centromeric plasmid pYS14 expressing HA_3Hrd3p. Microsomal membranes were prepared from these transformants and analysed by BN. Der3/Hrd3 protein complex I does not show any molecular mass shift on the BN-PAGE when both E2´s Ubc1p and Ubc7p are absent (Fig. 14A, lanes 4, 8 black arrowhead). This indicates that the two soluble ubiquitin conjugating enzymes Ubc1p and Ubc7p are not the part of the Der3/Hrd3 protein complex I. As observed in Fig 14A lane 6, when Der3p is missing, a weaker signal of the same molecular mass of the Hrd3p complex appears. It indicates that at this position on BN-PAGE there might be two Hrd3p containing protein complexes. One of which is independent of Der3p. Alternatively, Hrd3p remains partially associated with the main complex even after removal of Der3p.

A second ER E3 ligase that has been characterized is Doa10p. The enzyme is located in the ER membrane and is responsible for turnover of Ubc6p and Ste6p* but not CPY* (Swanson et al., 2001; Huyer et al., 2004). To test whether Doa10p is also part of the Der3p/Hrd3p complex I, microsomal membranes of wild type and Δ*doa10* cells were analysed by BN-PAGE (Fig. 14B). Comparison of Δ*doa10* and wild type cells shows that the

Der3p/Hrd3p complex I did not exhibit any molecular mass shift, suggesting that Doa10p is not part of the Der3p/Hrd3p complex I (Fig. 14B).

It has been shown that mutation of an essential cysteine (C399S) in the RING domain of Der3p leads to loss of function of the E3 and results in delayed ERAD (Bordallo and Wolf, 1999; Deak and Wolf, 2001). As the E3 activity of Der3p is required for its degradation in the absence of Hrd3p, this mutation should prevent its degradation and lead to high amounts of Der3p complex II in the absence of Hrd3p. I transformed Δ*der3* and Δ*der3*Δ*hrd3* cells with the centromeric plasmid pRS315 expressing a 3HA-tagged mutated version of Der3p (Der3C399S-HA_3). Microsomal membranes were prepared from these transformants and analysed by BN. Both Der3p and HA antibodies were used for Western analysis. Mutated Der3C399S-HA_3p was able to replace wild type Der3p to form the Der3p/Hrd3p complex I (Fig. 15, lanes 1 and 3 black arrowhead). Noticeably, an accumulation of the Der3C399S-HA_3 complex II could be observed in the absence of Hrd3p (Fig.15, lanes 2 and 4 grey arrowhead). Consistent with previous data (Bordallo and Wolf, 1999; Deak and Wolf, 2001) that the C399S mutation in the absence of Hrd3p compromises degradation of Der3p, these results confirm that Der3p degradation requires the presence of a functional RING-H2 domain. However, the data suggests that the RING structure of Der3p does not influence complex formation.

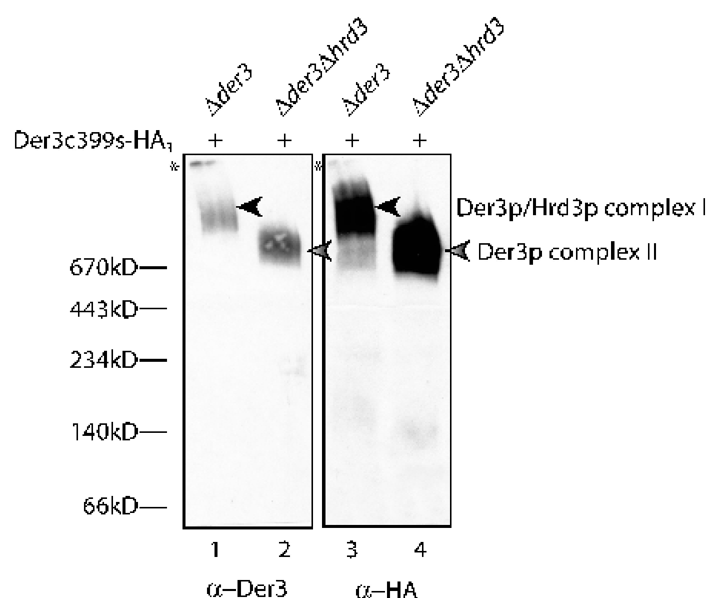


Figure 15. The Ring-H2 C399S mutation does not affect Der3p complex formation. BN-PAGE was performed as in Figure 13. The asterisk (*) denotes the bottom of the wells of the BN-PAGE. The Δ*der3* and Δ*der3*Δ*hrd3* cells expressing DER3c399s-HA_3 from plasmid pRS315 were analysed. For immune-detection, both anti-Der3 and anti-HA antibodies were applied.

To identify other possible protein components of the Der3/Hrd3 protein complexes, Coomassie staining protein bands from BN-PAGE were excised and analysed by MALDI-TOF. Due to the contamination of mitochondria membrane proteins, this approach failed (data not shown).

Der3p is an E3 ubiquitin protein ligase and it is hypothesized that Hrd3p may sense the requirement for Der3p ligase activity in the presence of substrates, perhaps by directly binding substrates, and signal through the Der3p transmembrane domain to activate Der3p function in a correct temporal and spatial manner (Gardner et al., 2000). As observed in Fig. 14A (Lanes 5, 6, 8), other than participating in a >700 kDa Der3p/Hrd3p complex I, Hrd3p is also part of a protein complex of around 140 kDa. How do these protein complexes containing Hrd3p, cooperate in ERAD? To address this question, a cycloheximide chase experiment was performed. Δ*hrd3* cells were transformed with the centromeric plasmid pXL3140 expressing a 3HA-tagged Hrd3p and a second plasmid overexpressing CPY*. Microsomal membranes were prepared from these transformants with the same amount of OD cells at different time points and analysed by BN. The gel was stained with Ponceau S to control for loading of equal amount of proteins. Western blots were analysed using anti-HA (to visualize Hrd3p) and anti-Der3p antibodies. In comparison with a stable amount of Der3p in the Der3/Hrd3 protein complex I, Hrd3p seems to be recruited to the Der3/Hrd3 protein complex I from a 140 kDa protein complex pool during the turnover of CPY* (Fig. 16).

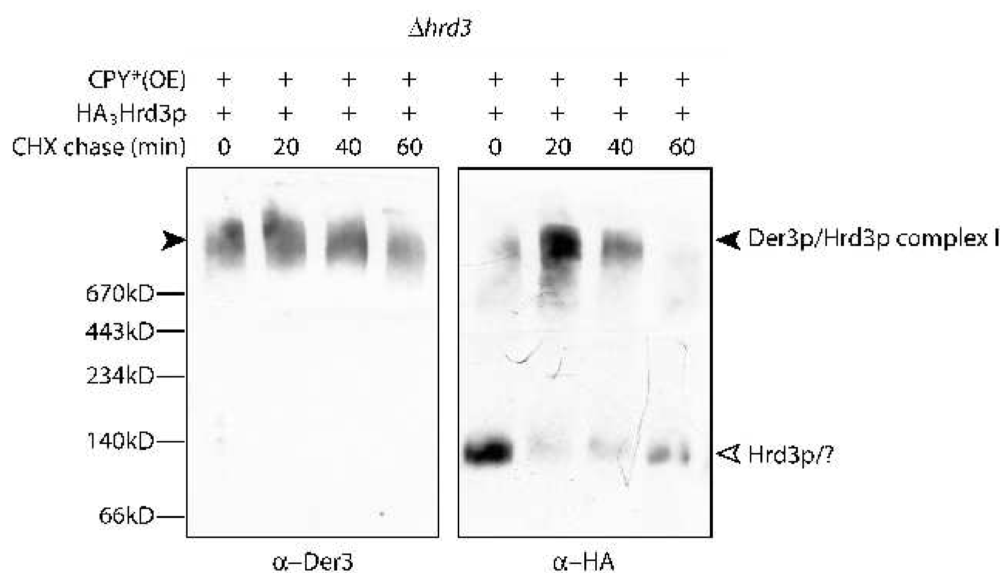


Figure 16. Hrd3p is dynamically involved in Der3p/Hrd3p complex I during turnover of CPY*. BN-PAGE was performed as shown in Figure 13. Cycloheximide (CHX; 100 µg/ml) was added and membrane extraction was performed at different time points. The Δ*hrd3* cells expressing pYS14 and pXL3141 were applied for analysis. The cells were grown at 30°C. For inducing the *CUP* promoter, 100 µM CuSO4 as a final concentration were added into the CM-media and incubated at 30°C for 3-5hrs. The immunoblots were probed with antibodies against HA (Sigma) and Der3p.

This kind of dynamic change of Hrd3p involved in different protein complexes may suggest another signalling function of Hrd3p. The Hrd3p signal in the 140 kDa complex is very strong

at zero point, which is different from the data shown in Fig. 14A lane 5. One possible explanation is that, due to overexpression of CPY*, more Hrd3p may be needed to bind CPY* to reduce ER stress.

To investigate whether Der3p/Hrd3p complexes could be a part of a retrotranslocon, I investigated whether the ERAD substrate CPY* is involved in this protein complex. As seen in Fig. 13, CPY* and Der3p seem to coexist in the same >700 kDa complex. However, due to the smeary signal of CPY*, it is hard to evaluate this blot. In order to exclude unwanted proteolytic degradation during native isolation and solubilization of microsomal membranes, the *Δprc1Δpep4Δprb1* strain, devoid of the vacuolar proteases carboxypeptidase Y, proteinase A, and proteinase B known to be responsible for unspecific proteolysis, was used. A centromeric plasmid pHIT341 expressing CPY* was transformed into these cells. Microsomal membranes were isolated from these transformants and analysed by BN (Fig. 17). Again, Der3p and CPY* were found to coexist in the 670 kDa and >700 kDa protein complexes. Besides, other CPY* signals were observed between 100 kDa and 234 kDa, suggesting that CPY* is also bound to other protein components of the membrane-associated ERAD machinery.

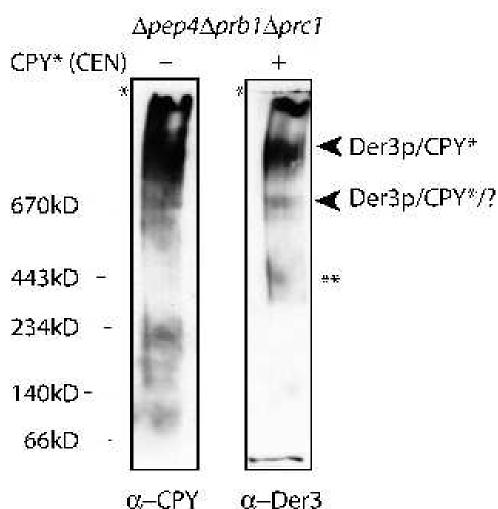


Figure 17 BN-PAGE determines the coexistence of Der3p and CPY* at high molecular mass protein complexes. The asterisk (*) denotes the bottom of the wells of the BN-PAGE. The cells *Δpep4Δprb1Δprc1* was transformed with a centromeric plasmid pHIT341 expressing CPY*. Microsomal membranes were isolated for these transformants and analysed by 4-12% BN-PAGE. The immunoblots were probed with antibodies against CPY and Der3p. Der3p and CPY* are coexisted at 670 kDa and >700 kDa protein complexes. The double asterisks (**) denote the cross-reaction.

Soluble and membrane bound substrates differ partly in their requirements for ERAD components. Degradation of all CPY* derivatives (soluble and membrane bound) (Taxis et al., 2003) depend on the E3 ligase Der3p. To find out whether the Der3p/Hrd3p complexes differ in size / composition when substrates containing a compactly folded domain or a transmembrane domain are present, CG* (CPY*-GFP), CT* (CPY* with the last transmembrane domain from Pdr5p) and CTG* (CPY*-transmembrane domain-GFP) were overexpressed in *Δprc1* strain and analysed, respectively. On BN-PAGE, Der3p and CG* (Fig. 18A), Der3p and CT* (Fig. 18B) and Der3p and CTG* (Fig. 18C) can be visualized in a

protein complex at around 700 kDa. Given that Der3p and Hrd3p coexist in this complex 700 kDa (Fig. 14, 15), this data shows that all these CPY* derivatives are bound to Der3p/Hrd3p complex I. Although CT*, CG* and CTG* have different molecular mass, no shift of protein complex is visible on BN-PAGE.

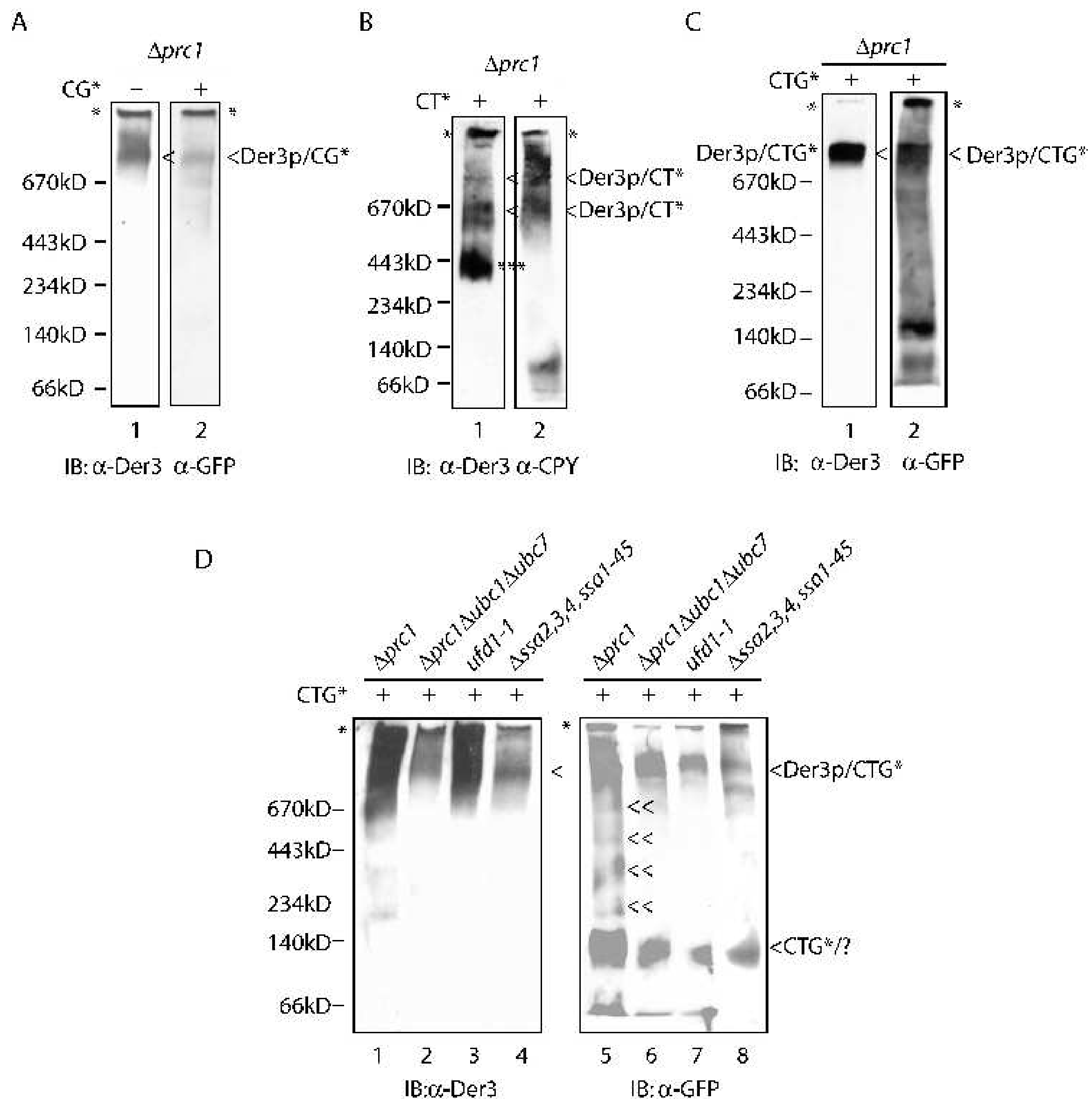


Figure 18. CG*, CT* and CTG* were found to be associated with Der3/Hrd3 protein complex I. BN assay was performed as Figure 13. The asterisk (*) denotes the bottom of the wells of the BN-PAGE. The triple asterisks denote the false signal of cross-reaction caused by anti-Der3p antibodies. The cells Δ*prc1* containing a 2μ plasmid pXL4242 expressing CG* (A), pCT67 expressing CT* under *TDH3* promoter (B) or PMA1 expressing CTG* under *TDH3* promoter (C) were analysed separately by BN following immunoblotting with anti-Der3 and anti-GFP antibodies. (D), the cells Δ*prc1*, Δ*prc1*Δ*ubc1*Δ*ubc7, ufd1-1* and Δ*ssa2, 3, 4, ssa1-45* containing PMA1 were analysed by BN following immunoblotting with anti-Der3 and anti-GFP antibodies.

In addition, I observed that, when CT* is overexpressed in Δ*prc1* cells, very little Der3p is left in >700 kDa protein complexes. Most Der3p and CT* are involved in another protein complexes at 670 kDa (Fig. 18B).

Ubc1p and Ubc7p are two soluble cytosolic ubiquitin conjugating enzymes; the polyubiquitination process is blocked in cells missing these two proteins. The cytosolic AAA ATPase Cdc48$^{Ufd1\text{-}Npl4p}$ complex was proposed to act as a pulling force on the cytosolic side of the ER membrane, to recognize and extract polyubiquitinated substrates from retrotranslocation channel out of the ER to the cytoplasm (Jarosch et al., 2002; Ye et al., 2003). *Ufd1-1* mutant cells show a strong inhibitory effect of this process. Ssa1p, Ssa2p, Ssa3p and Ssa4p belong to the cytosolic Hsp70 chaperone class which was supposed to help unfold the GFP domain of the ERAD substrate CTG* on the cytoplasmic side of ER membrane (Taxis et al, 2003). The degradation of CTG* was completely compromised in Δ*ssa2*, *3*, *4*, *ssa1-45* mutant cells (Taxis et al, 2003). In order to go into more detail of the functional machinery of Der3/Hrd3p complex in the retrotranslocation process, CTG* from plasmid PMA1 was overexpressed in ERAD mutants cells Δ*prc1*Δ*ubc1*Δ*ubc7, ufd1-1* and Δ*ssa2*, *3*, *4*, *ssa1-45*. Subsequently solubilized membrane protein complexes were analysed by BN. The coexistence of a Der3/Hrd3p complex and CTG* is not affected in these mutants (Fig. 18D). However, in comparison with multiple CTG* containing protein complexes identified in Δ*prc1* cells (Fig. 18D, lane 5 vs. lanes 6, 7, 8), it seems that the processing of CTG* in other ERAD deficient strains is prevented to a certain degree, if ubiquitination, pulling or unfolding on the cytosolic side of the ER is disturbed. This data is in agreement with our present belief that the interaction between Der3/Hrd3 protein complexes and ERAD target substrates takes place in the earlier steps of ERAD before ubiquitination and recognition on the cytosolic side of ER membrane. It may even be initiated before the complete formation of a functional retrotranslocon. In the case of CPY* derivatives, the Der3/Hrd3 protein complex, in addition to fulfilling the role of an E3 ligase complex, may even be part of the retrotranslocation channel.

3.2.2.2 Sec61p, a core component of the translocon or retrotranslocon

The central question that will be attacked in the forthcoming sections concerns the assembly and composition of the retrotranslocation channel of malfolded proteins out of the ER. Using CPY* as substrate, genetic experiments had provided evidence that the ER-channel protein Sec61p, required for protein import into the ER, also participates in the retrotranslocation of the ER degradation process (Plemper et al., 1997; Plemper et al., 1999) As protein import into the ER and protein export out of this organelle require a different

machinery, it was hypothesized that the retrotranslocation channel with Sec61p as the pore forming unit must have a different protein composition as compared to the Sec61p containing import channel (Kostova and Wolf, 2003). The plasticity of such channel forming processes with Sec61p as the core unit maybe supported by the fact that the Sec61p containing import channel disintegrates after protein translocation releasing free Sec61p in the membrane (Rapoport et al., 1996). As Der3p and Hrd3p form a complex in the ER membrane, the question arises if these components form a retrotranslocon on their own or if additional 'pore forming' proteins as Sec61p are recruited for retrotranslocation of soluble ER proteins. These potential complexes may play a role for retrotranslocation separately or cooperatively.

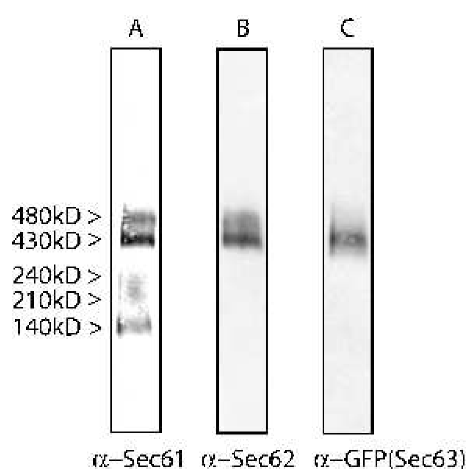


Figure 19. BN-PAGE of Sec61 protein complexes. W303-1C cells were transformed with pJK59 expressing Sec63-GFP. Microsomal membranes were isolated and analysed by a 5-13.5% BN-PAGE. Subsequently immunoblotting was performed with anti-Sec61 antibodies (A), anti-Sec62 antibodies (B) and anti-GFP antibodies for detecting Sec63p(C).

As shown in Fig. 14, in wild type cells the Der3p/Hrd3p complex appears at around 700 kDa molecular mass on BN-PAGE. The same pattern was visible when the detergents DBC and DDM were used instead of digitonin (data not shown). However, it was possible to observe stable Sec61p complexes in BN-PAGE only when digitonin extracts were used. Sec61p does not exist in free form but is mainly present in high-molecular-mass complexes of approximately 140 kDa, 210 kDa, 230 kDa, 430 kDa and 480 kDa (Fig. 19A). The higher molecular mass complexes of Sec61p (430 kDa and 480 kDa) are far more stable and less dynamic than the 230 kDa molecular complex. They remained rather constant upon changes of protein and detergent concentration (data not shown).

The yeast translocation complex of the ER is composed of two subcomplexes, the Sec61p complex consisting of Sec61p (41 kDa), Sss1p (9.1 kDa) and Sbh1p (8.7 kDa) and the Sec62/63 complex, consisting of Sec62p (30 kDa), Sec63p (73 kDa), Sec71p (31 kDa) and Sec72p (23 kDa). The association of both subcomplexes as a heptamer is required for protein translocation in yeast (Johnson and van Waes, 1999, Pilon et al., 1999). The 430 kDa and 480 kDa Sec61p complexes are common species of Sec61p containing complexes in yeast (Fraering et al., 2001) when yeast microsomes are analysed by BN-PAGE. Sec62p (Fig. 19B)

is part of both Sec61-containing 430 kDa and 480 kDa complexes while Sec63p is mainly a component of the 430 kDa complexes in the wild type (W303-1C) cells (Fig. 19C). The 430 kDa and 480 kDa Sec-complexes are possibly functional heptameric complexes in cells. However, it cannot be distinguished if they are solely a translocation complex, or a mixture of translocation- and possible retrotranslocation complexes. No Sec61p is visible together with the Der3/Hrd3p complex at around 700kD. If Sec61p is part of a different retrotranslocation complex, the failure to identify such a retrotranslocation complex via BN-PAGE might be due to a short half-life of the assembly. For the import channel (Panzner et al., 1995), it is known that the complex falls apart after translocation (Deshaies et al., 1991; Musch et al., 1992).

To test the idea that an increase of the assembly time of retrotranslocation complex could allow the identification of its components, five kinds of experiments were carried out. First, it is known that when Ubc proteins are missing, CPY* is hypo-ubiquitinated and is not pulled away from the ER. Therefore, the absence of E3 complexes or E2 enzymes like Ubc1p and Ubc7p might cause a blockage of the retrotranslocation channel by the retrotranslocating protein. Second, overexpression of CPY* might affect the dislocation in two ways. On one hand, it may induce formation of more retrotranslocons, and on the other, it could lead to an overloaded retrotranslocation, giving rise to a blockage of the retrotranslocon. Third, induction of malfolded proteins by DTT initiates the unfolded protein response (UPR) (Travers et al, 2000; Friedländer et al., 2000) and many proteins involved in ERAD are upregulated. Fourth, since a mutated Sec61 protein, Sec61-2p, is itself a substrate of ERAD at 38°C (Biederer et al., 1996; Bordallo et al., 1998), one could investigate changes of Sec61-2p and catch retrotranslocation intermediates by testing the turnover of Sec61-2p. Finally, by using a special “slippery” substrate (Zhang and Coffino, 2004) such as CPY*GAr215 (see section 3.1.3), one might be able to catch protein complexes in a retrotranslocation state and thereby identify the components of the retrotranslocon.

I attempted to block the ER retrotranslocation channel and isolate it as a whole by using yeast deletion strains defective in Der3p, Hrd3p or Ubc1p and Ubc7p, containing normal or high expression levels of the ERAD substrate CPY*. However, there was no visible difference of Sec61p containing complexes on BN-PAGE isolated from these ERAD mutants as compared to wild type cells (Fig. 20A lane 2 and data not shown). However, when microsomal membranes of Δ*ubc1*Δ*ubc7* cells overexpressing CPY*-HA_3 were analysed by BN, the CPY*HA_3 signal was found at multiple locations along the blot (Fig. 20A lane 1). Following a second dimensional analysis by SDS-PAGE, CPY*HA_3 could be observed bound to >700 kDa Der3p complex I (Fig. 20B black arrowheads). Moreover, two CPY*HA_3 signals

between 234 kDa and 443 kDa seem to be involved in Sec61p complexes (Fig. 20B white arrowheads). Since disturbance of the ubiquitination process leads to a block in retrotranslocation of malfolded proteins from the ER to the cytosol and since the translocation process of secretory proteins from the cytosol to the ER lumen is not affected in cells defective in ubiquitination enzymes, this data suggests that lumenal CPY*HA$_3$ might be bound and retrotranslocated via these Sec-complexes. In addition, in accordance with SDS-PAGE protein analysis (Fig. 20B), a dominant signal of free CPY*-HA$_3$ could be detected around 70 kDa by BN-PAGE (Fig. 20A lane 1). No clue could be found that Sec61p and Der3/Hrd3p complexes are involved in the same protein complexes.

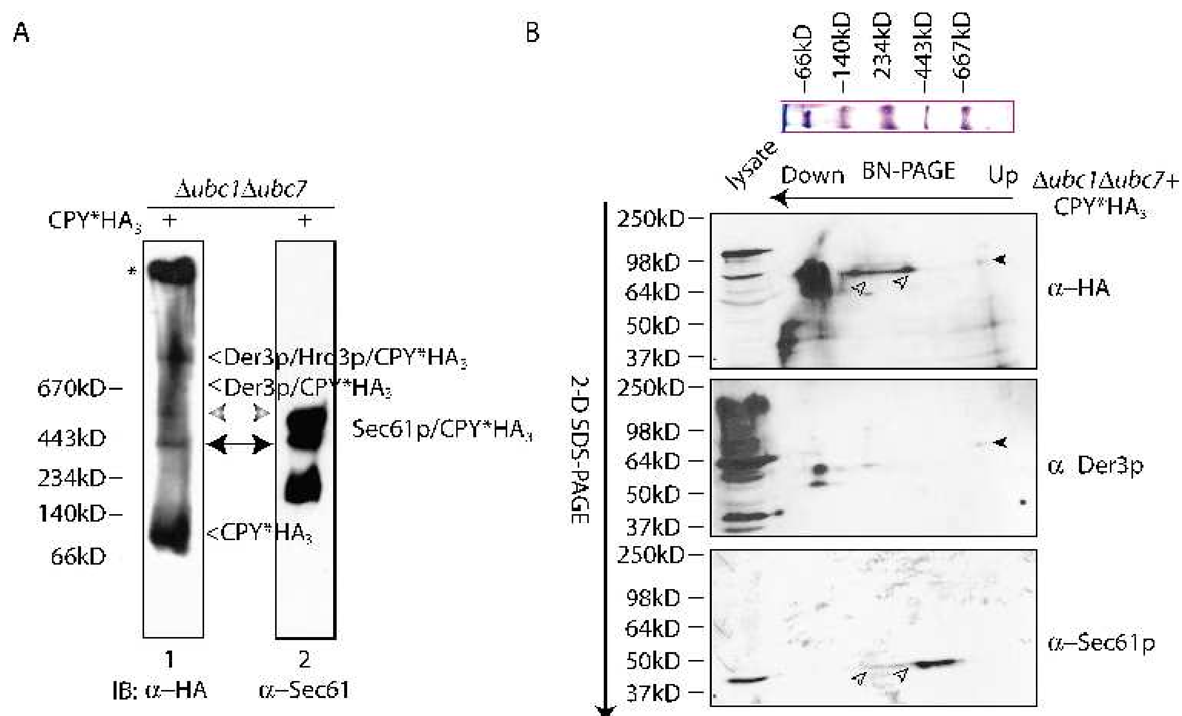


Figure 20. BN-PAGE uncovers CPY*HA$_3$ as being associated with Sec61 protein complexes in Δ*ubc*1Δ*ubc7* cells. Δ*ubc1*Δ*ubc7* cells were transformed with pCT51 expressing CPY*HA$_3$ under *CUP* promoter. The *CUP* promoter was induced as in Figure 16. The cells were then disrupted by glass beads. Microsomal membranes were analysed by a 5-13.5% BN-PAGE (A). Afterwards, BN-gel slices were excised and applied to 9% SDS-PAGE (B). White arrowheads show the co-existence of CPY* and Sec61p in two protein complexes. Black arrowheads show the co-existence of CPY*HA$_3$ and Der3p in >700 kDa protein complex. The asterisk (*) denotes the bottom of the wells of the BN-PAGE. Immunoblotting was performed with anti-HA, anti-Der3 and anti-Sec61 antibodies.

Accumulated aberrant ER resident proteins activate the unfolded protein response (UPR), which in turn upregulates the expression of UPR target genes encoding both protein of the import machinery such as Kar2p, which assists in refolding of denatured proteins in the ER, and proteins of ERAD machinery, promoting the turnover of disassembled or unfolded

proteins. Der1p, Der3p, Cue1p and Kar2p had been demonstrated to be ERAD components induced by UPR (Knop et al., 1996; Travers et al., 2000; Friedländer et al., 2000; Sidrauski et al., 2002). To test possible changes of a retrotranslocon under UPR conditions, wild type cells (W303-1C) containing genomically expressed CPY* were treated with 2mM DTT to induce the UPR, crude microsomal membranes were isolated from these cells at different time points and analysed by BN (see 2.2 methods). As seen in Fig. 21C lane 7, Kar2p migrated as a 140 kDa complex on BN-PAGE immunoblots. Interestingly, after inducing UPR with 2 mM DTT, an increasing amount of the 140 kDa Kar2p containing complex was observed, and concomitantly, an additional 70 kDa signal of free Kar2p could be observed (Fig. 21C, lane7 vs. lane 8 and 9).

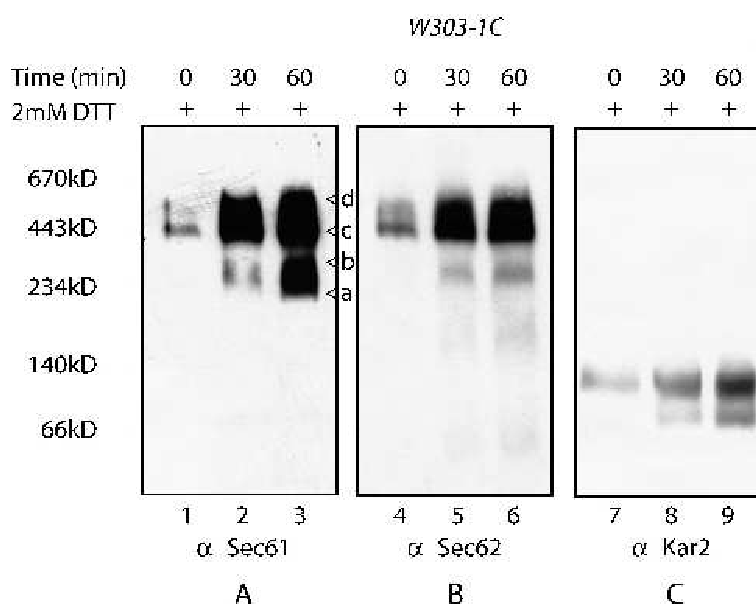


Figure 21. BN-PAGE following UPR induction of Sec61p, Sec62p and Kar2p. W303-1C cells were grown in YPD at 30°C. For inducing UPR, the cells were treated with 2mM DTT for 0, 30 or 60 min. Then, microsomal membranes were analysed by a 5-13.5% BN-PAGE. Proteins were transferred onto a PVDF membrane and were probed and reprobed with antibodies against Sec61 (A), Sec62 (B) and Kar2 (C).

This data suggests that the 140kDa signal may be dimerized Kar2p which increases following a successful UPR induction. However, under similar conditions, the level and location of Der3p complexes was not altered (data not shown). Conversely, the amount of two Sec61-Sec62 containing complexes of 430 kDa (c) and 480 kDa (d) and Sec61p containing complexes of 210 kDa (a) and 230 kDa (b) increased dramatically (Fig. 21A and B). It is interesting to note that 210 kDa-Sec61 complex (a) (Fig. 21A vs. Fig. 21B) is nearly devoid of Sec62p. In consistence with the data published on mRNA levels (Friedländer et al., 2000; Travers et al., 2002), it is determined by BN-PAGE that protein levels of Kar2p, Sec61p and

Sec62p are upregulated by UPR. However, this data does not provide information regarding the function (translocation or retrotranslocation) of the observed protein complexes.

Der1p is a very sensitive UPR target protein and its level can be easily enhanced by a temperature shift. Der1p acts in ERAD of ER-resident soluble substrates (Knop et al., 1996; Hitt and Wolf, 2004a,b). Due to poor antibodies, it is hard to detect Der1p under normal conditions. To investigate whether Der1p is part of protein complexes connected to retrotranslocation, wild type cells containing a genomically expressed *DER1-HA* were incubated at 25°C. After treating cells with DTT for 1 hour to induce unfolded protein response, microsomal membranes from these cells were analysed by BN. Interestingly, besides the 30-kDa monomer, a variety of complexes containing Der1p-HA (230 kDa, 430 kDa, 480 kDa and 670 kDa complexes) were identified (Fig. 22 lane 1). It is unclear at present what the higher molecular mass complexes of Der1p represent. Sec61p is present in two of these complexes (430 kDa and 480 kDa) (Fig. 22 lane 2). One could speculate that Der1p might be recruited to Sec-complexes or Der3/Hrd3 complexes (Fig. 22 lane 1 vs. lane 2) and function together in the retrotranslocon process when cells are under stress.

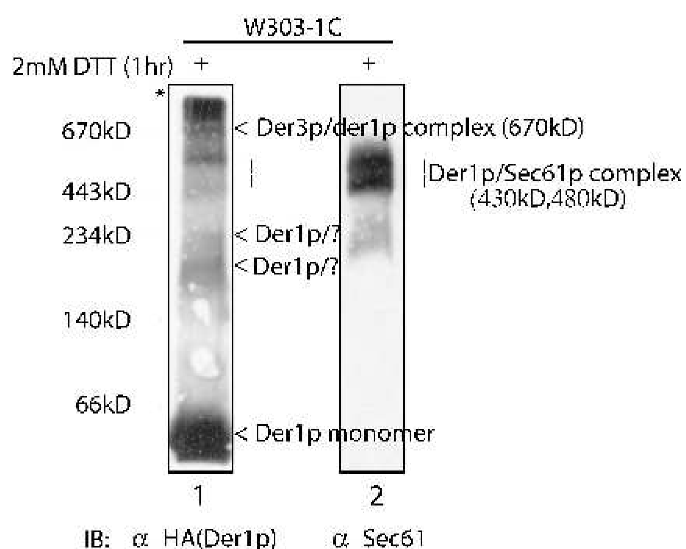


Figure 22. BN-PAGE of Der1p-containing protein complexes The asterisk (*) denotes the bottom of the wells of the BN-PAGE. W303-1C cells containing a genomically expressed DER1-HA were grown at 25°C until OD_{600} of the cells was around 0.7. Then the cells were treated with 2mM DTT for one hour. Isolated microsomal membranes were analysed by a 5-13.5% BN-PAGE. Proteins were transferred onto a PVDF membrane and were probed and reprobed with antibodies against HA (1) and Sec61p (2) for detecting Der1p and Sec61p.

One mutation in *SEC61* leading to the allele *Sec61-2* affects the stability of the Sec61 protein in yeast at 38°C, but does not lead to severe translocation defects when analysed at 25°C *in vitro* (Sommer and Jentsch, 1993). To gain a better understanding of the role of Sec61p complexes in the retrotranslocation process, cells endogenously expressing *sec61-2* were grown at 30°C, and then shifted to 38°C. It was hoped to visualize the behaviour of Sec61p complexes during their own retrotranslocation. Microsomal membranes from cells grown at 38°C were analysed by BN. It was found that the two dominant 430 kDa and 480

kDa Sec61p complexes formed as in wild type cells at 30°C. After cells were shifted to 38°C for one hour, the 430 kDa and 480 kDa complexes seemed to disassemble and merged to a higher position on BN-PAGE (Fig. 23). The results indicate a change in complex assembly under these conditions. Sec61-2p seems to reassemble with other proteins for its own turnover at 38°C. It is known that Sec61-2p is degraded very fast at 38°C (Plemper et al., 1997). However, the BN results do not clearly show a decrease of Sec61-2p within two hours. Due to experimental condition, large amount of cell culture (300 ml) was directly shifted from 30°C to 38°C. Hence, the temperature of cell culture increases slowly, leading to gradual response of cell to higher temperature, which may delay the degradation of Sec61-2p.

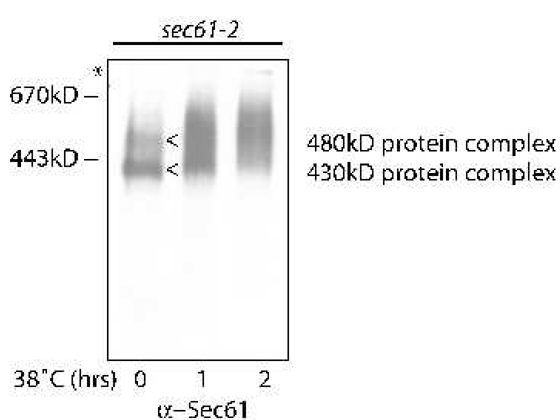


Figure 23. Sec61p complexes change their position when Sec61-2p containing complexes are analysed at restrictive temperature of 38°C. The asterisk (*) denotes the bottom of the wells of the BN-PAGE. Mutant *sec61-2* cells were grown at 30°C and shifted to 38°C for indicated time point before membrane extraction. BN-PAGE and Western analysis were performed as in Figure 20. Sec61-2p was detected by using Sec61 antibodies.

All these experiments indicate complex formation of known membrane components involved in ERAD (Der1p, Der3p, Hrd3p) as well as the existence of several high molecular mass complexes containing Sec61p. They do not answer yet the question of the retrotranslocation complex as a whole. This will only become possible if this complex can be caught in action while retrotranslocating a malfolded protein substrate. A good candidate to achieve this goal seemed to be CPY*GAr215, which was processed but not degraded by the proteasome (see section 3.1.3, Fig. 8). The explanation for this behaviour comes from experiments using an ODC-GAr chimera as substrate of the proteasome. The Gly-Ala (GA) repeat is "slippery" on the proteasome, preventing the ATPase ring of the 19S regulatory particle from pulling the substrate into the 20S core proteasome for degradation (Zhang and Coffino, 2004). This also seems to be true for the CPY* molecule containing Gly-Ala repeat, preventing it from being digested and pulled out completely out of the ER (Fig. 8). In order to know if the retrotranslocation channel becomes blocked by this construct and if any intermediates with the retrotranslocation channel are visible via BN-PAGE, an experiment was performed to analyse Sec61p and Der3p containing protein complexes in the presence of CPY*GAr215. A centromeric plasmid pZK129 expressing CPY*GAr215HA$_3$ was

transformed into the Δ*prc1* strain. As a control, a centromeric plasmid pHIT341 expressing CPY* and an empty vector plasmid pRS316 were used as well. CPY*GAr215HA$_3$ containing protein complexes migrated at molecular masses of 140 kDa, 430 kDa and >700 kDa, respectively (Fig. 24 lane 1,4). The 140 kDa and 430 kDa complexes appear to be dominant. Since a 430 kDa Sec61 protein complex co-localizes with CPY*GAr215HA$_3$ (Fig. 24 lane 7), it may suggest that Sec61p act during retrotranslocation. However, the 430 kDa Sec61p signal was also found in cells containing CPY* or empty plasmid (Fig. 24 lane 7 vs. lanes 8 and 9).

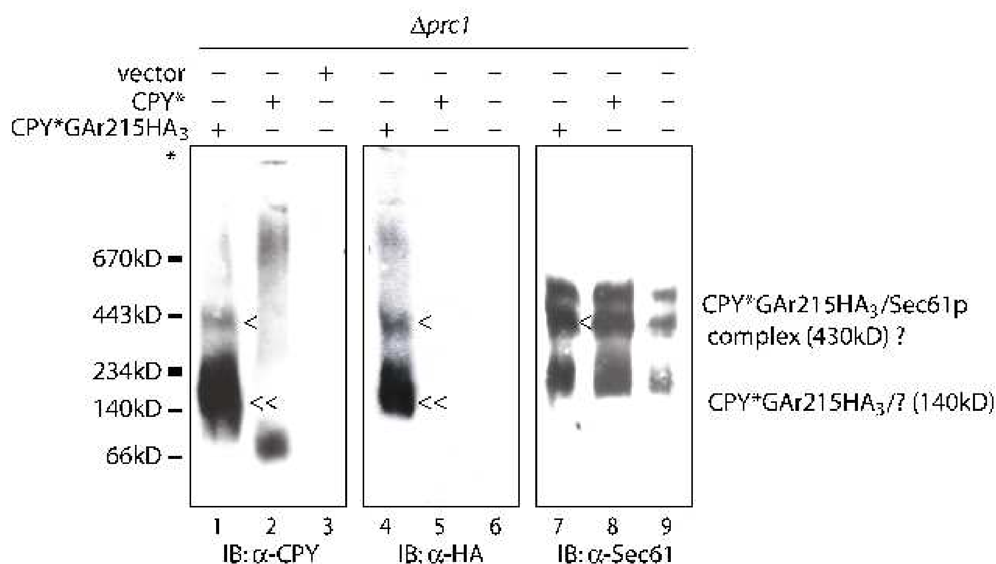


Figure 24. CPY*GAr215HA$_3$ is associated with 430kD Sec61 protein complexes. The asterisk (*) denotes the bottom of the wells of the BN-PAGE. BN-PAGE was performed as in Figure 13. Δprc1 cells containing pZK129 expressing CPY*GAr215HA$_3$ (Lanes 1,4,7), pHIT341 expressing CPY* (Lanes 2,5,8) or an empty plasmid pRS316 (Lanes 3,6,9) were analysed for complexes, respectively. Western analysis was done with antibodies against CPY, HA and Sec61 as indicated.

However, as only CPY*GAr and not CPY* was found at a position where also Sec61p was visible (430 kDa) (Fig. 24 lane 1 vs. lane 7), the question arose whether this was due to complex formation of CPY*GAr215 and Sec61p caused by blockage of the retrotranslocation channel. In addition, if the proteasomally processed intermediate of CPY*GAr215 were to coexist with the Sec61p complex (Fig. 8), this would be a strong argument for blockage of the channel by CPY*GAr215. Furthermore, as retrotranslocation substrates, all CPY* species must be N-glycosylated. To answer these questions, an *E*ndo H in-gel-digestion experiment was performed. After staining with Coomassie Blue G-250, the BN-PAGE pieces around 430 kDa were excised from the gel and digested with Endo H at 37°C overnight. Thereafter the digested gel pieces were subjected to a SDS-PAGE and their identity were determined by Western blot analysis. As shown in Fig. 25, *E*ndo H digestion leads to shift of both, full

length and intermediates of CPY*GAr215 to lower molecular masses, indicating that both species of CPY*GAr215 were glycosylated. This is proof that both species had entered the ER and must be on their way out of retrotranslocation. The coexistence of Sec61p with both glycosylated full length and intermediates of CPY*GAr215 in this excised gel slices verified by 2-D western analysis (Fig. 25), indicates an interaction between Sec61p complex and ERAD processing of CPY*GAr215. As a model, one might propose that, when CPY*GAr215 is targeted for retrotranslocation, the proteasome and the trimeric Cdc48$^{Ufd1\text{-}Npl4p}$ complex can not grasp the slippery Gly-Ala sequence and therefore the undigested intermediate of CPY*GAr215 slides back to channel. The channel is therefore interacting with both species of CPY*GAr215.

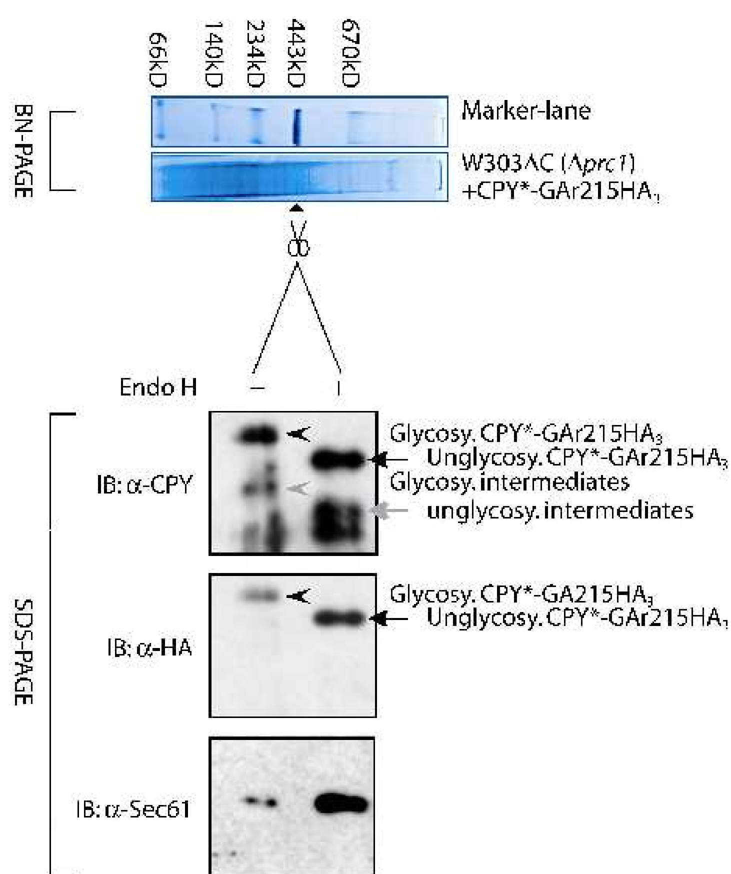


Figure 25. Both, full length and intermediates of CPY*GAr215HA$_3$ are associated with 430kDa Sec61 protein complexes. BN-PAGE was performed as in Figure 13. W303ΔC (Δ*prc1*) cells containing pZK129 expressing CPY*GAr215HA$_3$ were analysed by BN-PAGE. In gel digestion with or without *E*ndo H was performed as described in section 2.2.4.10.3.3. Then the materials were subjected to 9% SDS-PAGE. Immunoblotting was done with antibodies against CPY, HA and Sec61p.

To further investigate the processing of CPY*GAr215, a cycloheximide decay experiment was performed and both BN and SDS-PAGE followed by Western analysis were applied to analyse protein turnover and possible conformational or compositional changes of the various complexes. Interestingly, three CPY*GAr215 containing protein complexes were visible: Der3p-CPY*GAr215 protein complex at around 700 kDa (Fig. 26A lanes 1-3 and lanes 7-9), Sec61-CPY*GAr215 protein complex at around 430 kDa (Fig. 26A lanes 1-3 and

lanes 4-6) and CPY*GAr215 containing protein complex at 140 kDa. Sec61p is also found at 140 kDa at zero time point (Fig. 26 lane 4). CPY*GAr215 has a molecular mass of 89 kDa.

Figure 26. CPY*GAr215 is associated with a 430kDa protein complex containing Sec61p. The asterisk (*) denotes the bottom of the wells of the BN-PAGE. W303ΔC (Δ*prc1*) cells expressing CPY*GAr215 from pZK102 were applied for cycloheximide decay experiments. Microsomal membranes from these transformants at each time point were collected and analysed by 4-12% BN-PAGE. The subsequent Western analysis was performed with antibodies against Der3p, CPY and Sec61p (A). In the meanwhile, 2.5OD cells from each time points were lysed and analysed by SDS-PAGE. The Western analysis was performed with antibodies against CPY and Sec61p levels at each time point were determined as loading controls (B).

This 140 kDa protein complex may suggest the co-existence of a single Sec61p and CPY*GAr215. Consistent with SDS-PAGE and Western analysis (Fig. 26B), the immune signal of CPY*GAr215 in the 140 kDa protein complex disappeared after 90min, while that of 430 kDa protein complex remained stable after 90min (Fig. 26A lanes 1-3 and lanes 4-6). This data leads to the following model: during cycloheximide chase, proteins are targeted to the retrotranslocation machinery from the ER lumen. The Gly-Ala sequence slows down the

turnover of CPY*GAr215 and leads to a processed 78 kDa intermediate released by the proteasome. It is possible that full length CPY*GAr215 and the degradation intermediate are "sticking" in the retrotranslocation channel and waiting for further processing. Due to this, the ERAD pathway might be kinetically disturbed when CPY*GAr215 is present in the cells, leading to longer interaction of the substrate with the retrotranslocation channel. This proposal is supported by the growth defect visible in *Sec61-2* cells expressing the CPY*GAr215 (see section 3.1.3, Fig. 11). Sec61p available for growth-promoting protein import is diminished when a certain amount of Sec61p is engaged with the malfolded CPY*GAr215 for export.

3.2.2.3 Cdc48p is actively involved in the retrotranslocation process

The AAA-ATPase Cdc48 protein complex has been widely studied. Gel filtration experiments show that ER membrane associated p97 (yeast orthologue Cdc48p) forms two complexes of around 670 kDa, named p97/p47 and $p97^{Npl4/Ufd1}$ (Meyer et al., 2000; Bruderer et al., 2004). It was shown that p97/p47 (Cdc48/Shp1 in yeast) is involved in ER-Golgi membrane fusions (Meyer et al., 2002) and the action of $Cdc48^{Npl4/Ufd1p}$ complex is essential for dislocation of ERAD-target proteins (Bays et al., 2001a; Ye et al., 2001; Braun et al., 2001; Jarosch et al., 2002; Rabinovich et al., 2002; Gnann et al., 2004; Medicherla et al., 2004; Richly et al., 2005). $Cdc48^{Npl4/Ufd1p}$ complex is able to recognize polyubiquitinated substrates emerging from the retrotranslocon and potentially acts as a motor to pull them out of the ER membrane or mobilize them from the ER membrane and subsequently transfer them to 26S proteasome for degradation. At the time of these experiments it was not clear how this protein complex is recruited to the ER membrane and works independent of the putative retrotranslocon. In order to address this question, I applied BN-PAGE to detect whether Cdc48p is part of protein complexes located in the ER membrane of cells (Δ*prc1*) expressing CPY*GAr215. Five Cdc48p containing protein complexes can be observed (Fig. 27A, marked with arrows). Except for the 100 kDa Cdc48p monomer, no significant difference was visible when these complexes were identified with 5mM ATP added to guarantee a functional AAA ATPase Cdc48p complex (Fig. 27A). The two 670 kDa signals might indicate Cdc48p/Sph1p and $Cdc48^{Npl4/Ufd1}$ protein complexes, respectively. By performing the same experiment in *pre1-1* cells expressing CPY*GAr215 or an empty plasmid, the Cdc48p protein complex 1 was found to be associated with the Der3/Hrd3 protein complex I (>700 kDa) independently of CPY*GAr215 (Fig. 27B lanes 1,3 vs. lanes 2,4). Cdc48 protein complex 5 was found to be associated with the 430 kDa Sec61 protein complex only in cells expressing CPY*GAr215 (Fig. 27B lanes 3,5 vs. lanes 4,6). This data might suggest the formation of Cdc48p/Der3p and Cdc48p/Sec61p protein complexes in the cells. It needs to be emphasized that the molecular

masses seen in BN-PAGE cannot give any information on complex composition. However, as the Sec61p banding pattern (430 kDa and 480 kDa) never changes under any experimental condition, it could still be argued if Cdc48p and CPY*GAr215 just show up occasionally at the same position of Sec61p.

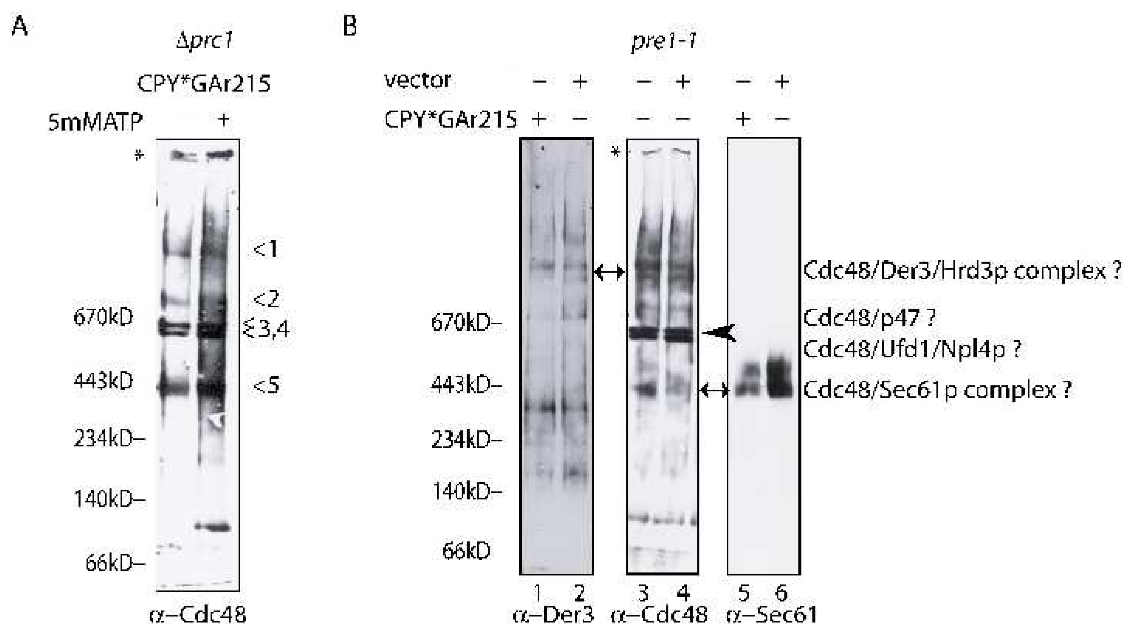


Figure 27. Different Cdc48p containing protein complexes can be identified by BN-PAGE. A midi 4-12% BN-PAGE was applied. The asterisk (*) denotes the bottom of the wells of the BN-PAGE. Five Cdc48p associated membrane protein complexes were found. A Cdc48/Der3 protein complex (>700kD) and Cdc48 protein complexes around 670kD were found on BN-PAGE. In addition, Cdc48 was found to be associated with the 430kD Sec61 protein complexes. A, Microsomal membranes of W303ΔC (Δ*prc1*) cells expressing CPY*GAr215 from pZK102 were solubilized with or without addition 5mM ATP in all buffers prior to BN-PAGE and later for immunoblotting with antibody against Cdc48p. B, Microsomal membranes of *pre1-1* cells expressing CPY*GAr215 from pZK102 or containing empty vector pRS316 were solubilized and subjected to BN-PAGE. Immunoblotting was performed with antibody against Der3p, Cdc48p and Sec61p, respectively.

3.3 Protein interactions of the ERAD machinery determined by co-immunoprecipitation

Blue Native analysis uncovered a variety of protein complexes containing protein components involved in ERAD. Three kinds of protein complexes containing ERAD proteins seem to be the most important: an > 700 kDa Der3p/Hrd3p complex I (See section 3.2.2.1), 430 kDa Sec61p containing complexes (See section 3.2.2.2) and 670 kDa Cdc48p complexes (See section 3.2.2.3). These complexes could also be detected together with the ER substrate CPY* or CPY*GAr215, suggesting that they might be part of a retrotranslocation channel.

However, due to limitation of BN analysis, information on components of the complexes cannot be completely obtained solely by this method. Moreover, because BN-PAGE is limited in terms of true molecular masses of the membrane protein complexes, changes in composition within the various complexes are hard to visualize. To obtain further insight into the mutual dependence of the observed protein complexes and associated proteins, I therefore undertook co-immunoprecipitation experiments. 6-Aminocaproic acid is a mild organic compound used in Blue Native electrophoresis and the effect of 750 mM 6-aminocaproic acid in protein solubilization experiments is comparable to that of 500 mM NaCl (Schägger and von Jagow, 1991). To preserve the weak interactions of the retrotranslocation complex, I used 6-aminocaproic acid in IP buffer for facilitating the solubilization of membrane proteins. 1% digitonin solubilized microsomal membranes were used as input.

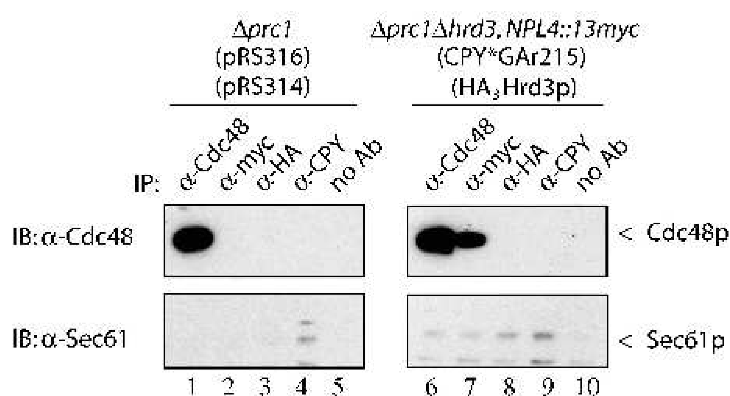


Figure 28. Interaction between Sec61p and Hrd3p, $Cdc48^{Ufd1\text{-}Npl4p}$ is specific upon the expression of CPY*GAr215. Co-immunoprecipitation experiments were performed with indicated antibodies as described in section 2.2.4.11. CPY*GAr215 was expressed under the *TDH3* promoter from pXL3164. The cells were collected when the OD_{600} of the cells reaches around 1.0. 5% Precipitates were analysed by immunoblotting with the antibodies against Sec61p and Cdc48p.

Sec61p seems to be a sticky protein that easily binds unspecifically to many antibodies (Hitt unpublished results and personal communication). To test whether the antibodies used in this study unspecifically bind to Sec61p, a preliminary experiment was performed. As shown in Fig. 28, the solubilized microsomal membranes of Δ*prc1* cells, containing two empty vectors (pRS314 and pRS316), were analysed by IP with the indicated antibodies followed by immunoblotting with antibody against Sec61p. In parallel, Δ*prc1*Δ*hrd3* cells containing an endogenous Npl4-Myc_{13} fusion and expressing HA_3Hrd3p from pXL3140 and CPY*GAr215 from pZK102 were used as positive control. Equal precipitation efficiency in both yeast strains was controlled by immunoblotting with antibodies against Cdc48p (Fig. 28). As can be seen, no unspecific binding of Sec61p to affinity purified antibodies against Cdc48p, Myc,

HA was found (Fig. 28 lanes 1, 2, 3 vs. lanes 6, 7, 8). But an unspecific binding of Sec61p to polyclonal antibody against CPY (Fig. 28 lane 4 vs. lane 9) exists.

3.3.1 Interaction between Der3p/Hrd3p complex and Cdc48p complex

Der3p/Hrd1p and Hrd3p are the first ER anchored complex known to exert ubiquitin ligase activity. Its activity is required for retrotranslocation of CPY* and a variety of other malfolded proteins. BN analysis identified a Der3p/Hrd3p/substrate protein complex and other larger Cdc48p/Der3p protein complexes (Fig. 27B). To investigate the mutual dependence of the interactions between Der3p, Hrd3p and Cdc48p, I analysed Δ*hrd3*Δ*prc1* cells expressing HA_3Hrd3p plus CPY* and Δ*hrd3*Δ*prc1* cells expressing HA_3Hrd3p plus vector pRS316 by co-immunoprecipitation (co-IP) with the indicated antibodies. Immunoblotting with specific antibodies showed that HA_3-Hrd3p, Der3p and CPY* were coprecipitated with Cdc48p (Fig. 29A, lane 1).

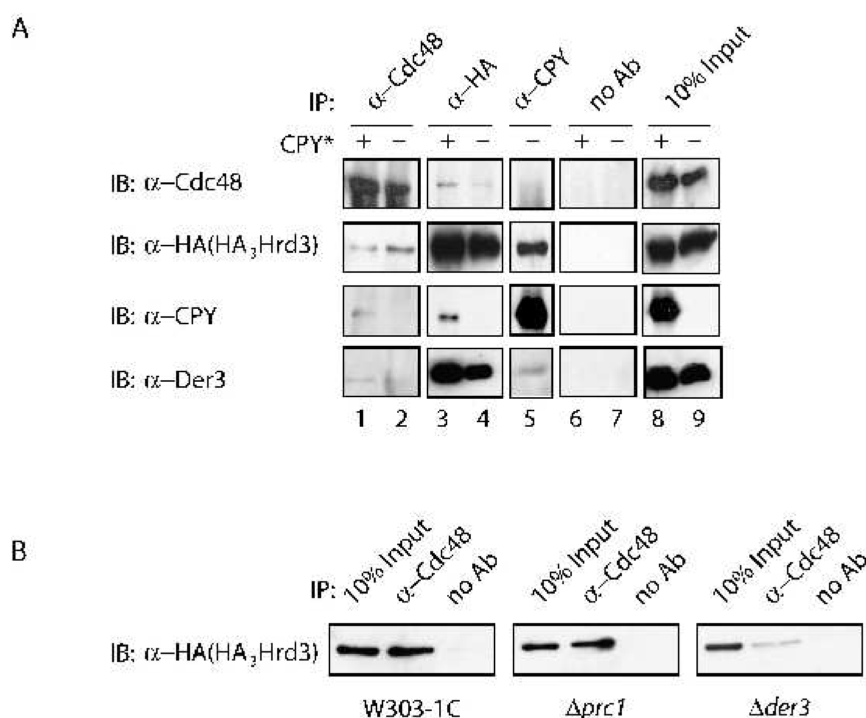


Figure 29. Cdc48p binds to the Der3p/Hrd3p protein complex Co-IP experiments were performed with indicated antibodies as described in section 2.2.4.11. A, solubilized membrane proteins (Input) and precipitated proteins from Δ*prc1*Δ*hrd3* cells (+) expressing HA_3-Hrd3p from pXL3140 and CPY* from pHIT341 or Δ*prc1*Δ*hrd3* cells (–) containing pXL3140 and an empty vector pRS316 were analysed with a 5-15% SDS-PAGE and immunoblotted with the indicated antibodies. B, Co-IP experiments of Cdc48p was performed from yeast strains W303-1C, Δ*prc1* and Δ*der3*. Analysis of input and precipitate was done by immunoblotting with HA antibody.

Der3p and HA_3-Hrd3p could be co-precipitated with Cdc48p independently of the presence of CPY* (Fig. 29A, lane 2). Albeit weakly, Cdc48p was in turn co-precipitated with HA_3-Hrd3p independent of the CPY* substrate (Fig. 29A, lanes 3,4), indicating that Cdc48p might be able to interact with Hrd3p. In accordance with the data shown by Gardner 2000, the interaction between Hrd3p and Der3p could be easily detected (Fig. 29A, lanes 3,4). In agreement with the data from BN, this data suggests that Cdc48p may be recruited to the Der3p/Hrd3p protein complex. Next, I investigated the possible interaction of CPY* with ER membrane proteins. I found that both Der3p and Hrd3p could be co-precipitated with CPY* (Fig. 29A, lane 5). It was shown by Schuberth and Buchberger (2005) that a HA-tagged CPY* could precipitate Cdc48p by using antibody against HA. However, I wasn't able to reproduce their results (Fig. 29A lane 5). One possible explanation for this discrepancy is that polyclonal anti-CPY antibody may not be able to precipitate Cdc48p. In line with BN results, this data shows that an interaction exists between the Der3p/Hrd3p ubiquitin ligase complex, the substrate CPY* and the Cdc48 protein complex in wild type cells (Δ*hrd3*Δ*prc1* strain expressing HA_3Hrd3p). Interestingly, compared with wild type cells W303-1C, Cdc48p could still precipitate a little HA_3Hrd3p in mutant cells deficient in E3 ligase Der3p (Fig. 29B). In agreement with BN analysis, this data confirms that Hrd3p might have other functions in ERAD other than being interacting partner of Der3p.

*3.3.2 Sec61p interacts with Hrd3p and Cdc48p upon expression of CPY*GAr215*

BN analysis showed that the ERAD substrate CPY*GAr215 co-localizes with Sec61p and Cdc48p in single protein complexes around 430 kDa, suggesting that Sec61p might be part of a retrotranslocon channel (Fig. 24, 25, 26, 27). However, these proteins could also accidentally migrate at the same position. To examine if Sec61p can indeed participate in ERAD, co-immunoprecipitation experiments were performed to detect possible interactions between Sec61p and ERAD associated protein components upon expression of CPY*GAr215. For this purpose, yeast strains Δ*prc1*Δ*hrd3* (expressing HA_3Hrd3p plus CPY*GAr215, Fig 30 lanes 1-4, 9) and Δ*prc1*Δ*der3*Δ*hrd3* (expressing HA_3Hrd3p plus CPY*GAr215, Fig. 30 lanes 5-8, 10) were analysed. Cdc48p could precipitate the Der3p/Hrd3p ubiquitin ligase complex and both full length and the intermediate of CPY*GAr215 in wild type cells (Δ*prc1*Δ*hrd3* strain expressing HA_3Hrd3p) (Fig. 30 lane 1). Meanwhile, HA_3Hrd3p could also efficiently precipitate Cdc48p, Der3p and both forms of CPY*GAr215 in wild type cells (Δ*prc1*Δ*hrd3* strain expressing HA_3Hrd3p) (Fig. 30 lane 3). Most interestingly, a small amount of Sec61p was co-immunoprecipitated by Cdc48p and HA_3Hrd3p in wild type cells (Δ*prc1*Δ*hrd3* strain

expressing HA_3Hrd3p) (Fig. 30, lanes 1,2,3). This data suggests the formation of a potential Cdc48p/Der3p/Hrd3p/substrate/Sec61p protein complex. Although Cdc48p could precipitate efficiently both, full length and intermediates of CPY*GAr215, CPY antibodies could not precipitate Cdc48p, but the Der3p/Hrd3p protein complex and a low level of Sec61p (Fig. 30 lane 2). Using HA antibodies, 3HA tagged CPY*GAr215 did not precipitate Cdc48p either (data not shown). The partially processed and retrotranslocated CPY*GAr215 may not have the capacity to co-precipitate Cdc48p.

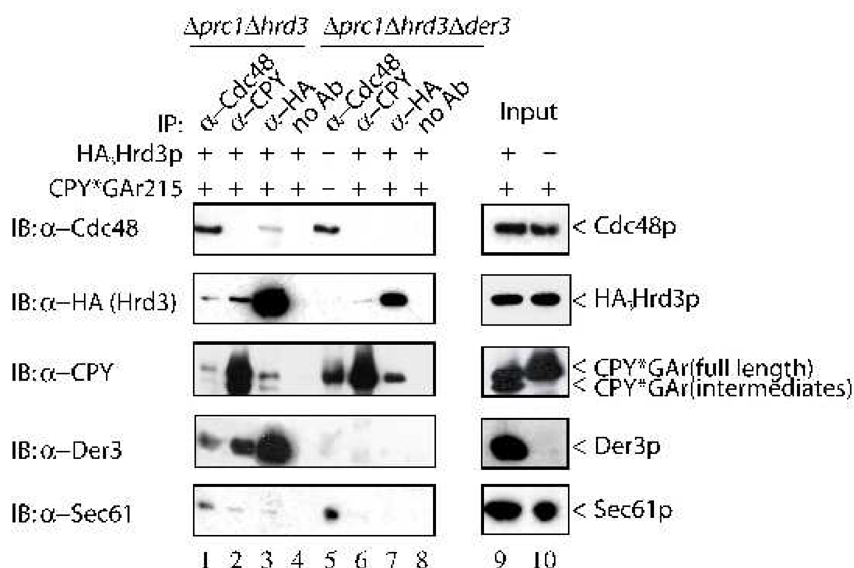


Figure 30. Interaction between a Der3p/Hrd3p/Cdc48p/CPY*GAr215 protein complex and Sec61p Co-IP experiments were performed with indicated antibodies as described in section 2.2.4.11. Solubilized membrane proteins (Input) and precipitated proteins from cells Δ*prc1*Δ*hrd3* (lanes 1-4, 9) and cells Δ*prc1*Δ*der3*Δ*hrd3* (lanes 5-8, 10) expressing HA_3Hrd3p from pXL3140 and CPY*GAr215 from pZK102 were analysed with 5-15% SDS-PAGE. Western blotting was performed with indicated antibodies.

It was recently demonstrated that the Cdc48/p97p ATPase complex acts on substrates by binding directly to unmodified and presumably unfolded segments of a polypeptide chain. For further binding, it required the poly-ubiquitin chains attached to the substrate (Ye, et al., 2003). Hence, I investigated whether deletion of one of the components of the Der3p/Hrd3p ubiquitin ligase complex affects the interaction between Cdc48p and CPY*GAr215. In accordance with Ye's data, the experiment showed that CPY*GAr215 was still co-precipitated with Cdc48p in the absence of Der3p (Fig. 30 lane 5), although processing of CPY*GAr215 is prevented in this ERAD deficient strain (Fig. 8B). However, when Der3p is missing, the interaction between HA_3Hrd3p and Cdc48p and the interaction between HA_3Hrd3p and Sec61p were interrupted (Fig. 30 lanes 5 and 7, respectively). This data suggests that Der3p is necessary for the interaction of Hrd3p with possible retrotranslocation

channel protein Sec61p. Compared with Fig. 29B, the loss of interaction between Cdc48p and Hrd3p in Der3p deficient cells could be due to the special structure of Gly-Ala repeats within CPY*GAr215. Moreover, in line with BN data, HA_3Hrd3p could still interact with non-processed ER lumenal CPY*GAr215 (Fig. 30 lanes 6,7). this data indicates that Hrd3p might act in the process of substrate recognition in the ER lumen independent of Der3p.

Altogether, this data suggests that three different membrane protein complexes may exist in cells: Cdc48p/Sec61p/Der3p/Hrd3p/CPY*GAr215, Cdc48p/Sec61p/CPY*GAr215 and Cdc48p/Hrd3p/CPY*GAr215, which might indicate different stages of the retrotranslocation process. Cdc48p could mediate the association between substrates and the ubiquitin ligase complex via directly or indirectly interacting with one of them. In support of the BN results and the physiological growth test, co-IP experiments show weak interaction between Sec61p and ERAD proteins such as Hrd3p and Cdc48p and substrate CPY*GAr215, suggesting that Sec61p might be an ER retrograde transport channel protein.

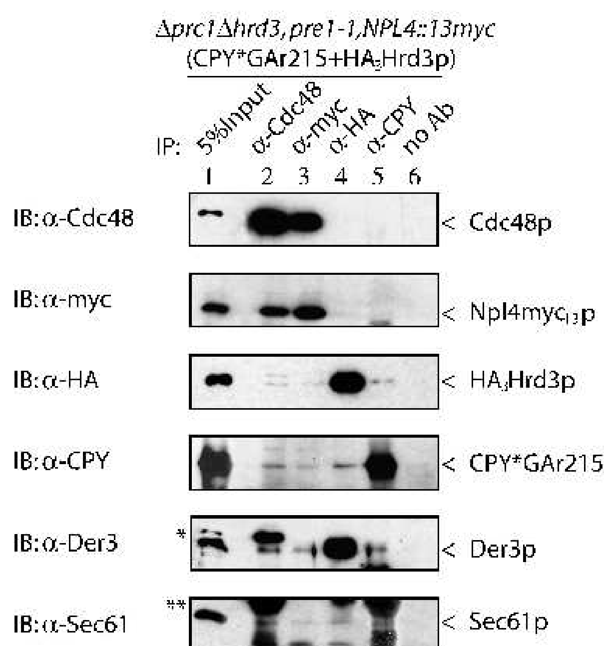


Figure 31. Sec61p was identified to interact with the Der3p/Hrd3p protein complex and $Cdc48^{Ufd1-Npl4p}$ also in proteasome mutant *pre1-1* cells expressing CPY*GAr215. Co-immunoprecipitation experiments of Cdc48p, Npl4p-Myc_{13}, HA_3-Hrd3p and CPY*GAr215 were performed from *pre1-1*cells deleted in CPY (Δ*prc1*) and expressing endogenous Npl4-13Myc, into which pXL3164 expressing CPY*GAr215 under the *TDH3* promoter and pXL3140 expressing HA_3Hrd3p were transformed. 5% Input and precipitates were analysed with 5-15% SDS-PAGE and the proteins were immunoblotted with the indicated antibodies. * designates cross reaction of immune signal. ** designates IgG.

To investigate possible compositional changes of the observed protein complexes, co-immunoprecipitation experiments were performed in proteasome mutant cells (Δ*prc1*, *pre1-1*) expressing an endogenous Cdc48 complex partner protein 13myc tagged Npl4. I anticipated that, due to an overload of CPY*GAr215 in proteasome mutant cells, more stages of the retrotranslocation process may be visible and active retrotranslocation protein complexes may be enriched. However, no differences in the interacting partners of Cdc48p were observed

(Fig. 30 lane 1 vs. Fig. 31 lane 2). The same interacting partners of Cdc48p and Npl4p-Myc13 were confirmed when both affinity purified polyclonal antibody against Cdc48p and monoclonal antibody against Myc were used for immunoprecipitation (Fig. 31 lanes 2 and 3). Additionally as shown in Fig. 31, in *pre1-1* cells, as well as in proteasome wild type cells (Fig. 30), the interaction between Cdc48p, Npl4p-Myc13, HA_3Hrd3p, Der3p, CPY*GAr215 and Sec61p might suggest that these proteins are able to constitute a functional protein complex for the retrotranslocation of substrates out of the ER. As found in ERAD mutant Δ*der3* cells, the interaction between HA_3Hrd3p and the $Cdc48^{Ufd1\text{-}Npl4p}$ complex was interrupted in *pre1-1* mutant cells, since neither Cdc48p and Npl4p-Myc13 could be co-precipitated with HA_3Hrd3 (Fig. 30 lane 7 & Fig. 31 lane 4) and less HA_3Hrd3p could be co-precipitated by either Cdc48p or Npl4-Myc13. Very little HA_3Hrd3p was precipitated by Cdc48p or Npl4p-Myc13 in *pre1-1* mutant cells (Fig. 31 lanes 2, 3) as compared to in wild type cells (Fig. 30 lane 5), possibly indicating that Hrd3p could be one of the important regulatory components for recruiting a functional retrotranslocation protein complex. Noticeably, in *pre1-1* mutant cells, Sec61p was still able to be co-precipitated with both, Cdc48p and other known ERAD membrane associated proteins as Hrd3p and ERAD target substrate CPY*GAr215 (Fig. 31 lanes 4, 5), indicating that, there might be also differences in constitution of a retrotranslocation complex between proteasomal mutants cells and mutants cells deleted in Der3p.

4. Discussion

Cells prevent aggregation of malfolded proteins in the ER by maintaining a strict quality control system. When this process fails in helping proteins acquire their native conformations, these malfolded proteins undergo a process called ER associated degradation (ERAD). Malfolded proteins are retained in the ER, retrotranslocated across the endoplasmic reticulum membrane back into the cytosol, where they are ubiquitinated and finally degraded by the proteasome (Hiller et al., 1996). During or immediately following retrotranslocation malfolded proteins are ubiquitinated via an ER-membrane resident ubiquitin-protein ligase (E3) complex composed of Der3p/Hrd1p (E3) and Hrd3p (Bordallo et al., 1998; Deak et al., 2000; Gardner et al., 2001). In some cases a second E3 ligase, Doa10p, operates (Swanson et al., 2001, Huyer et al., 2004). Liberation of the ubiquitinated proteins from the ER membrane is achieved by the trimeric $Cdc48^{Ufd1p/Npl4p}$ complex (Jarosch et al., 2002; Ye et al., 2001; Ye et al., 2003). Finally they are handed over to the proteasome via the UBA-UBL domain containing proteins Dsk2p and Rad23p (Medicherla et al., 2004). The identity and composition of the retrotranslocation channel allowing malfolded proteins to cross the ER membrane is under intensive debate. The multi-membrane spanning proteins Der1p, Der3p and Doa10p, are considered candidates for the formation of a retrotranslocation channel due to their pore-forming capacity. Because these membrane proteins are substrate-specific (Hampton et al., 1996; Bordallo et al., 1998; Plemper et al., 1998,1999b; Swanson et al., 2001; Huyer et al., 2004), some believe that different ERAD substrates might be retrotranslocated through different channels. However, as both Der3p and Doa10p are two E3 ubiquitin ligases, they seem more likely to be modulators of a retrotranslocation channel. Up till now, most experimental evidence obtained from the study of various ERAD substrates suggested that protein export from the ER is mediated by a channel containing Sec61p, the central component of the ER-protein import channel (Wiertz et al., 1996b; Pilon et al., 1997, 1998; Plemper et al., 1997; Schmitz et al., 2000, 2004; Imai et al., 2005). For ER-import of proteins, the trimeric Sec61p complex has first to interact with a tetrameric Sec62p/Sec63p protein complex to form an active import channel (Pilon et al., 1997). In yeast, genetic data indicate an interaction between Sec61p and the ERAD components Hrd3p and Der3p (Plemper et al., 1999a). This leads to the assumption that the export channel might be formed in a similar manner by the interaction of a free trimeric Sec61p complex with membrane proteins known to be part of the ERAD machinery (Römisch, 1999). Although bidirectional traffic (import and export) using the same channel forming entity may seem economical for the cell's

standpoint, there is no experimental proof indicating that Sec61p is also involved in the export process. To gain further insight into ERAD-related processes, I investigated whether Sec61p participates in retrotranslocation from the ER using a biochemical approach.

To identify the ERAD retrotranslocation complex, Blue Native electrophoresis was introduced in our research field, which allows a rapid assessment of the state of membrane protein complexes under various conditions. Blue Native electrophoresis has been successfully used for analysis of mitochondrial protein complexes and their composition (Schägger and von Jagow, 1991). It was also successfully employed for isolation of the yeast OST complex located in ER membrane (Knauer and Lehle, 1999). Due to presence of ten hydrophobic transmembrane domains, Sec61p is the most difficult membrane protein to be solubilized from the ER. Hence, Sec61p is a reliable indicator of solubilization efficiency of microsomal membranes. Following by optimised experimental conditions, Sec61p was observed in five distinct protein complexes of 140 kDa, 210 kDa, 230 kDa, 430 kDa and 480 kDa. Fraering and his colleagues also observed the same 430 kDa and 480 kDa Sec61p protein complex in 2001. The 210 kDa and 230 kDa Sec61p complex species as well as 140 kDa complex are three additional complexes found in my study. These results indicated that the modified protocol functions as expected.

By Blue Native analysis, a stable ER resident E3 ligase complex was found at > 700 kDa containing both Der3p and Hrd3p. Two E2 conjugating enzymes Ubc1p and Ubc7p, and another ER membrane resident E3 enzyme Doa10p do not participate in this protein complex. Different ERAD substrates, such as soluble CPY*, CG* and the transmembrane CTG* were found to be associated with this protein complex. However, there was no shift in molecular mass of the Der3p/Hrd3p/Substrate complex compared to the Der3p/Hrd3p complex observed in cells that do not express ERAD substrates. A possible explanation is that BN analysis is limited in showing the correct molecular mass of membrane protein complexes. Instead, the position at which such complexes migrate might mainly depend on their overall shape. In line with the genetic observations that Der3p is degraded quickly in Δ*hrd3* cells, the Der3p/Hrd3p complex dissociates completely on BN-PAGE when the interacting partner Hrd3p is missing and another Der3p containing protein complex II appears at 670 kDa. In addition, the formation of the > 700 kDa Der3p/Hrd3p complex I is independent of the E3 ligase function of Der3p because a RING-H2 C399S mutation within Der3p, which inactivates the enzyme, does not disturb the formation of the Der3p/Hrd3p complex. This data suggest that Hrd3p might stabilize Der3p through interaction with the transmembrane domain of Der3p. Interestingly, in agreement with genetic data that E3 enzyme activity is necessary for self-

ubiquitination of Der3p (Deak et al., 2001), all Der3C399Sp is found in Der3p containing protein complex II after Der3p/Hrd3p dissociates in Δ*hrd3* cells. In cells, Hrd3p and Der3p are expressed at similar levels (Gardner et al., 2000), indicating a stoichiometric relationship between Der3p and Hrd3p. However, the finding by Plemper et al. (1999) that Der3p overexpression suppresses the destabilizing effect of Δ*hrd3* suggested that Hrd3p might have additional roles in ERAD. BN analysis found two Hrd3p containing protein complexes: One is associated with Der3p and migrates at >700 kDa, the other migrates at 140 kDa. The existence of two different Hrd3p containing protein complexes might represent two different functions of Hrd3p. Provided that the small Hrd3p complex at 140 kDa exists in cells, deletion of *DER3* would lead to destruction of Der3p/Hrd3p protein complex I (>700 kDa) with a parallel increase in the 140 kDa complex. In support of this assumption, BN analysis found higher amounts of 140 kDa Hrd3p complex in Δ*der3* cells. Co-immunoprecipitation experiments suggest that the partner Hrd3p in the 140 kDa complex may be the ERAD lumenal substrate CPY*. Since most of the Hrd3p sequence resides in the ER lumen (Plemper et al., 1999; Saito et al., 1999), one could speculate that the lumenal domain of Hrd3p senses the requirement for the ubiquitin ligase Der3p following interaction with lumenal substrates and signals through the Der3p transmembrane domain to activate Der3p' ligase function (Gardner et a., 2000). In this study, I demonstrate that Der3p and Hrd3p exist as a protein complex located in the ER membrane and might be a central part of the retrotranslocation complex. However, no Sec61p was found to be associated with this E3 protein complex. It is very likely that the active retrotranslocation complex is very dynamic and dissociates quickly. Convincing proof for Sec61p's participation in retrotranslocation may only come from experiments performed under conditions in which malfolded proteins jammed the retrotranslocation channel or the kinetics of retrotranslocation were significantly reduced.

We reasoned that an ERAD substrate containing a strongly folded domain could be used to block the retrotranslocation channel protein as shown for blocking protein import into mitochondria (Eilers and Schatz, 1986). Thus, in the beginning of this study, a lot of attention was given to the construction of CPY* fusion proteins containing compactly folded (DHFR, BPTI or Rhodanese) or heavily glycosylated domains (Suc2p). I anticipated that a compactly folded or heavily glycosylated domain fused to CPY* may jam the retrotranslocation channel, hence allowing the identification of the channel components. However, this idea failed as the fusion proteins CPY*-DHFR, CPY*-BPTI, CPY*-Rhodanese and CPY*-Suc2p were efficiently degraded in wild type cells. Consistent with my results, Fiebiger and Tirosh (2002) found that the presence of fully folded domains does not impair ER degradation: both EGFP-

tagged and DHFR-tagged Class I MHC HCs substrates were dislocated out of the ER while EGFP and DHFR retained their folded state (Fiebiger et al., 2002; Tirosh et al., 2002, 2003). More recently, removal of N-linked glycans from ERAD substrates was found to occur after extraction but prior to proteasomal destruction (Blom et al., 2004; Katiyar et al., 2004), suggesting that heavily glycosylated proteins can leave the ER.

A 238 amino acid long GlyAla repeat in the EBNA1 protein blocks proteasomal degradation and helps EBNA virus escape immune detection (Leight and Sugden, 2000; Levitskaya et al., 1995,1997; Heessen et al., 2003). GlyAla repeats introduced into the proteasomal substrate ornithine decarboxylase (ODC) inhibit full degradation of the enzyme and give rise to a degradation intermediate of ODC (Zhang and Coffino, 2004). It seems that GlyAla repeats act as stop transfer signals for substrate processing by the proteasome and generate proteolytic fragments that end at the site of GlyAla insertion. Zhang and Coffino (2004) suggested that "slippery" GlyAla repeats couldn't be grabbed by the proteasome due to an "idling" of the 19S cap. It is, then, plausible that an ERAD substrate containing GlyAla repeats may, similarly, be stalled at the ATPases of the Cdc48p complex or of the 19S cap of the proteasome. This "idling" may increase the length of time that the substrate remains associated with the retrotranslocon and offer the possibility to isolate the ER retrotranslocation complex. A 215 amino acid long GlyAla sequence was, previously, introduced to CPY* at position 433, followed by a 3HA-tag (unpublished results). CPY*GAr215HA$_3$ is synthesized as a 100 kDa protein. In wild type cells, this protein is not completely degraded, but processed to a 78 kDa intermediate lacking the C-terminus. Processing of CPY*GAr215HA$_3$ depends on the ubiquitin conjugating enzymes Ubc1p and Ubc7p, the E3 ligase Der3/Hrd1p and the proteasome (C. Alberola (2003) Diplomarbeit, Universität Stuttgart; T. Bohnacker (2004), Diplomarbeit, Universität Stuttgart). I determined that both full length CPY*GAr215HA$_3$ and the processed intermediate are N-glycosylated, indicating that both species have entered the ER. CPY*GAr215HA$_3$ contains the CPY signal sequence and undergoes post-translational translocation into the ER. I replaced the CPY signal sequence of CPY*GAr215HA$_3$ with the signal sequence derived from invertase (Suc2p) which is co-translationally translocated. Both species of co-translationally translocated protein are also glycosylated, indicating that GlyAla repeat inserted to the CPY* does not interfere its translocation into the ER. Interestingly, not only full size CPY*GAr215HA$_3$ is localized to the ER, but also the 78 kDa intermediate is found inside this organelle. This finding suggests that the Cdc48 complex is unable to dislocate the 78 kDa CPY*GAr intermediate from the ER membrane. Consecutively, the incompletely processed intermediates may slip back into the

retrotranslocation channel. At the same time, the channel might be engaged with full length CPY*GAr215HA$_3$ as well. If the import channel protein Sec61p is also involved in protein export, this prolonged interaction of CPY*GAr215HA$_3$ (full length and 78 kDa intermediate) with a Sec61p containing retrotranslocon may, under certain conditions, lead to growth defects due to sub-optimal levels of ER-import channels. To address this issue, I did a growth test using temperature sensitive *sec61-2* cells. The *sec61-2* allele is lethal to cells above 38°C because it leads to the degradation of Sec61-2p (Plemper et al., 1999). Upon constitutive expression of CPY*GAr215 in a *sec61-2* strain, a slight but reproducible growth reduction was observed even at 35°C. This physiological data is in agreement with the idea that Sec61p is sequestered in retrograde transport channels. The hypothesis of a prolonged engagement with substrate is supported by the finding that, in wild type cells, glycosylated forms of both full length and intermediate of CPY*GAr215 migrate at 430 kDa on BN-PAGE. This 430 kDa complex contains Cdc48p and Sec61p. However, a Cdc48p, and CPY* containing complex does not appear when microsomal membranes of cells transformed with CPY* are analysed. Interestingly, I found that, upon cycloheximide chase analysis, CPY*GAr215 at 430 kDa remains stable over the duration of the chase while the signal at 140 kDa decreases quickly. This is in agreement with the idea that CPY*GAr215 block the retrotranslocon. However, Sec61p generally migrates at 430 kDa when analysed by BN electrophoresis. Hence, I wanted to know whether these proteins run accidentally at the same position. To address this question, I carried out co-immunoprecipitation experiments, to determine interactions among the AAA-ATPase Cdc48$^{Ufd1/Npl4p}$ complex, the E3 ligase complex Der3p/Hrd3p, the ERAD substrate CPY*GAr215 and Sec61p. Cytosolic Cdc48p and its partner Npl4p precipitated full-length and intermediate CPY*GAr215, Der3p, HA$_3$Hrd3p and Sec61p. Similarly, HA$_3$Hrd3p also precipitated full-length CPY*GAr215 and its 78 kDa intermediate, Der3p, Cdc48p and albeit weakly, Sec61p. The same proteins were co-precipitated when CPY antibodies were used. My study shows that a complex consisting of Sec61p, Der3p/Hrd3p, Cdc48$^{Ufd1-Npl4p}$ and substrate can be isolated both by BN-PAGE and co-immunoprecipitation. At this point, the stoichiometry of the retrotranslocation complex is completely unknown. Besides the components I have identified, the retrotranslocation complex may contain components of the ubiquitination machinery, such as Cue1p and Ubc7p.

During preparation of my thesis, two groups reported that Ubx2p, an integral ER membrane protein containing both a UBA and a UBX domain, mediates binding of Cdc48 to the ubiquitin ligases Der3p and Doa10p, and to ERAD substrates (Schuberth and Buchberger, 2005; Neuber et al., 2005). In addition, they found that Ubx2p and Cdc48p interact with

Der1p and Dfm1p, yeast homologues of the putative dislocation pore protein Derlin-1 (Hitt and Wolf, 2004; Lilley and Ploegh, 2004; Ye, et al., 2004). Therefore, Ubx2p may also play a role in the assembly of the functional retrotranslocon. In my study, Cdc48p precipitated Hrd3p independent of Der3p and substrate. In cells deleted in *UBX2*, Cdc48p interacted with Hrd3p to a certain degree, even though the interaction between Cdc48p and Der3p is lost (Neuber et al., 2005). These observations suggest that Cdc48p and Hrd3p interact before Ubx2p recruits Cdc48p to the E3 complex. Additionally, I have determined that Hrd3p interacts with CPY*GAr215 in Δ*der3* cells. Hrd3p is a transmembrane protein with only 43 amino acids in the cytosol and 767 amino acids resides in the ER lumen. Hence, Hrd3p might sense, via its large lumenal domain, the ER stress caused by malfolded proteins and signal to Cdc48p indirectly at an earlier stage of retrotranslocation.

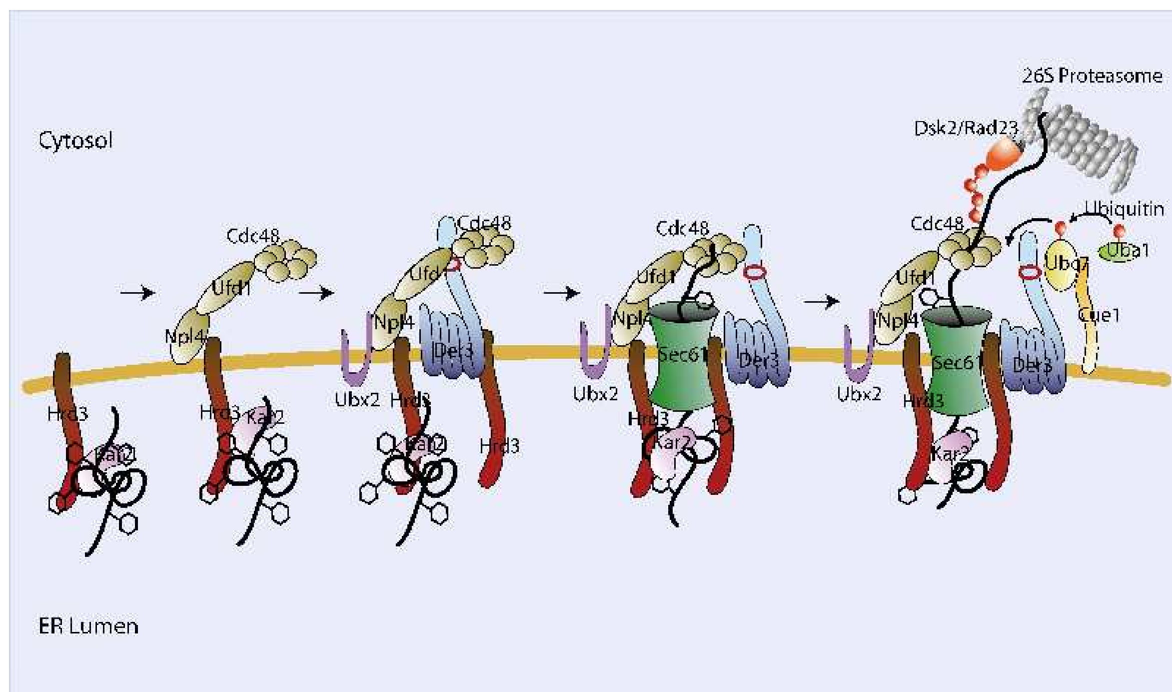


Figure 32. Retrotranslocation model proposed for ERAD It shows here a possible model of the retrotranslocation process of model substrate like CPY*.

Generally, ERAD can be broken down into four parts: quality control of malfolded proteins in the ER lumen and recognition of misfolding; delivery to the retrotranslocon; retrotranslocation across the ER membrane; extraction and degradation via the ubiquitin–proteasome system. This study focused on the identification of the retrotranslocation complex involved in ERAD. Using a substrate capable of blocking retrotranslocation, namely

CPY*GAr215. I performed a combination of an *in vivo* growth test and *in vitro* biochemical analyses (Blue Native electrophoresis and co-immunoprecipitation) to show that Sec61p may participate in retrotranslocation, as indicated earlier by genetic analysis (Plemper et al., 1999). This finding will have to be further substantiated by additional experiments in the future.

In light of my findings and published reports, I propose a model for the retrotranslocation of malfolded proteins from the ER (Fig. 32). 1. ERAD substrates associate with Hrd3p, which senses ER stress. 2. The cytosolic AAA-ATPase $Cdc48^{Ufd1/Npl4p}$ complex is recruited to the ER membrane indirectly by Hrd3p via an unknown co-factor. 3. Ubx2p mediates the association of the Cdc48p complex with a pre-existing Der3p/Hrd3p complex. 4. Sec61p joins the $Cdc48^{Ufd1/Npl4p}$-Ubx2p-Der3p/Hrd3p complex to form a mature retrotranslocation channel. At the same time, components of the ubiquitin system and the 26S proteasome are, also, recruited to the ER membrane.

References

Alam, S. L., Sun, J., Payne, M., Welch, B. D., Blake, B. K., Davis, D. R., Meyer, H. H., Emr, S. D., and Sundquist, W. I. (2004). Ubiquitin interactions of NZF zinc fingers. *Embo J* **23,** 1411-21.

Alberola, C. (2003). Der Einfluß von Gly-Ala Repeats am Ubiquitin-Proteasom-abhaengigen Abbau eines missgefalteten Proteins, CPY*. *In* "Institut für Biochemie". Universitaet Stuttgart, Stuttgart.

Ausubel, F. M., Kingston, R. E., Seidman, F. G., Struhl, K., Moore, D. D., Brent, R., and Smith, F. A. (1992). "Current Protocols in Molecular Biology." Greene, New York.

Bays, N. W., Gardner, R. G., Seelig, L. P., Joazeiro, C. A., and Hampton, R. Y. (2001a). Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER- associated degradation. *Nat Cell Biol* **3,** 24-9.

Bays, N. W., Wilhovsky, S. K., Goradia, A., Hodgkiss-Harlow, K., and Hampton, R. Y. (2001b). HRD4/NPL4 Is Required for the Proteasomal Processing of Ubiquitinated ER Proteins. *Mol Biol Cell* **12,** 4114-28.

Bazirgan, O. A., and Hampton, R. Y. (2005). Cdc48-Ufd2-Rad23: the road less ubiquitinated? *Nat Cell Biol* **7,** 207-9.

Bebök, Z., Mazzochi, C., King, S. A., Hong, J. S., and Sorscher, E. J. (1998). The mechanism underlying cystic fibrosis transmembrane conductance regulator transport from the endoplasmic reticulum to the proteasome includes Sec61beta and a cytosolic, deglycosylated intermediary. *J Biol Chem* **273,** 29873-8.

Beckmann, R., Spahn, C. M., Eswar, N., Helmers, J., Penczek, P. A., Sali, A., Frank, J., and Blobel, G. (2001). Architecture of the protein-conducting channel associated with the translating 80S ribosome. *Cell* **107,** 361-72.

Biederer, T., Volkwein, C., and Sommer, T. (1996). Degradation of subunits of the Sec61p complex, an integral component of the ER membrane, by the ubiquitin-proteasome pathway. *Embo J* **15,** 2069-76.

Biederer, T., Volkwein, C., and Sommer, T. (1997). Role of Cue1p in ubiquitination and degradation at the ER surface. *Science* **278,** 1806-9.

Blom, D., Hirsch, C., Stern, P., Tortorella, D., and Ploegh, H. L. (2004). A glycosylated type I membrane protein becomes cytosolic when peptide: N-glycanase is compromised. *Embo J* **23,** 650-8.

Bohnacker, T. (2004). Viral GlyAla repeats as a tool in ER associated protein degradation research. In "Institut fuer Biochemie". Universitaet Stuttgart, Stuttgart.

Bordallo, J., Plemper, R. K., Finger, A., and Wolf, D. H. (1998). Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol Biol Cell* **9,** 209-22.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72,** 248-54.

Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (2002). Role of the ubiquitin-selective CDC48(UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates. *Embo J* **21,** 615-21.

Brodsky, J. L., and McCracken, A. A. (1999). ER protein quality control and proteasome-mediated protein degradation. *Semin Cell Dev Biol* **10,** 507-13.

Cabral, C. M., Liu, Y., and Sifers, R. N. (2001). Dissecting glycoprotein quality control in the secretory pathway. *Trends Biochem Sci* **26,** 619-24.

Chiang, H. L., and Schekman, R. (1991). Regulated import and degradation of a cytosolic protein in the yeast vacuole. *Nature* **350,** 313-8.

Corsi, A. K., and Schekman, R. (1996). Mechanism of polypeptide translocation into the endoplasmic reticulum. *J Biol Chem* **271,** 30299-302.

Cronin, S. R., Khoury, A., Ferry, D. K., and Hampton, R. Y. (2000). Regulation of HMG-CoA reductase degradation requires the P-type ATPase Cod1p/Spf1p. *J Cell Biol* **148,** 915-24.

Dantuma, N. P., Heessen, S., Lindsten, K., Jellne, M., and Masucci, M. G. (2000). Inhibition of proteasomal degradation by the gly-Ala repeat of Epstein-Barr virus is influenced by the length of the repeat and the strength of the degradation signal. *Proc Natl Acad Sci U S A* **97,** 8381-5.

de Virgilio, M., Weninger, H., and Ivessa, N. E. (1998). Ubiquitination is required for the retro-translocation of a short-lived luminal endoplasmic reticulum glycoprotein to the cytosol for degradation by the proteasome. *J Biol Chem* **273,** 9734-43.

Deak, P. M., and Wolf, D. H. (2001). Membrane topology and function of Der3/Hrd1p as a ubiquitin-protein ligase (E3) involved in endoplasmic reticulum degradation. *J Biol Chem* **276,** 10663-9.

DeLaBarre, B., and Brunger, A. T. (2003). Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains. *Nat Struct Biol* **10,** 856-63.

Deshaies, R. J., Sanders, S. L., Feldheim, D. A., and Schekman, R. (1991). Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex. *Nature* **349,** 806-8.

Dreveny, I., Kondo, H., Uchiyama, K., Shaw, A., Zhang, X., and Freemont, P. S. (2004). Structural basis of the interaction between the AAA ATPase p97/VCP and its adaptor protein p47. *Embo J* **23,** 1030-9.

Dürr, G., Strayle, J., Plemper, R., Elbs, S., Klee, S. K., Catty, P., Wolf, D. H., and Rudolph, H. K. (1998). The medial-Golgi ion pump Pmr1 supplies the yeast secretory pathway with Ca2+ and Mn2+ required for glycosylation, sorting, and endoplasmic reticulum-associated protein degradation. *Mol Biol Cell* **9,** 1149-62.

Eilers, M., and Schatz, G. (1986). Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. *Nature* **322,** 228-32.

Elkabetz, Y., Shapira, I., Rabinovich, E., and Bar-Nun, S. (2004). Distinct steps in dislocation of luminal endoplasmic reticulum-associated degradation substrates: roles of endoplamic reticulum-bound p97/Cdc48p and proteasome. *J Biol Chem* **279,** 3980-9.

Ellgaard, L., and Helenius, A. (2003). Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol* **4,** 181-91.

Ellgaard, L., Molinari, M., and Helenius, A. (1999). Setting the standards: quality control in the secretory pathway. *Science* **286,** 1882-8.

Feldheim, D., and Schekman, R. (1994). Sec72p contributes to the selective recognition of signal peptides by the secretory polypeptide translocation complex. *J Cell Biol* **126,** 935-43.

Fiebiger, E., Story, C., Ploegh, H. L., and Tortorella, D. (2002). Visualization of the ER-to-cytosol dislocation reaction of a type I membrane protein. *Embo J* **21,** 1041-53.

Finger, A., Knop, M., and Wolf, D. H. (1993). Analysis of two mutated vacuolar proteins reveals a degradation pathway in the endoplasmic reticulum or a related compartment of yeast. *Eur J Biochem* **218,** 565-74.

Finke, K., Plath, K., Panzner, S., Prehn, S., Rapoport, T. A., Hartmann, E., and Sommer, T. (1996). A second trimeric complex containing homologs of the Sec61p complex

functions in protein transport across the ER membrane of S. cerevisiae. *Embo J* **15,** 1482-94.

Fraering, P., Imhof, I., Meyer, U., Strub, J. M., van Dorsselaer, A., Vionnet, C., and Conzelmann, A. (2001). The GPI transamidase complex of Saccharomyces cerevisiae contains Gaa1p, Gpi8p, and Gpi16p. *Mol Biol Cell* **12,** 3295-306.

Friedlander, R., Jarosch, E., Urban, J., Volkwein, C., and Sommer, T. (2000). A regulatory link between ER-associated protein degradation and the unfolded-protein response. *Nat Cell Biol* **2,** 379-84.

Frohlich, K. U., and Madeo, F. (2000). Apoptosis in yeast--a monocellular organism exhibits altruistic behaviour. *FEBS Lett* **473,** 6-9.

Gardner, R. G., Swarbrick, G. M., Bays, N. W., Cronin, S. R., Wilhovsky, S., Seelig, L., Kim, C., and Hampton, R. Y. (2000). Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. *J Cell Biol* **151,** 69-82.

Gilstring, C. F., and Ljungdahl, P. O. (2000). A method for determining the in vivo topology of yeast polytopic membrane proteins demonstrates that Gap1p fully integrates into the membrane independently of Shr3p. *J Biol Chem* **275,** 31488-95.

Gnann, A., Riordan, J. R., and Wolf, D. H. (2004). Cystic Fibrosis Transmembrane Conductance Regulator Degradation Depends on the Lectins Htm1p/EDEM and the Cdc48 Protein Complex in Yeast. *Mol Biol Cell* **15,** 4125-35.

Guthrie, C., and Fink, G. R. (1991). "Guide to Yeast Genetics and Molecular Biology." San Diego.

Haigh, N. G., and Johnson, A. E. (2002). Protein sorting at the membrane of the endoplasmic reticulum. *In* "Protein targeting, transport and translocation" (R. E. Dalbey and G. von Heijne, Eds.), pp. 74-106. Academic Press, London-New York.

Hamman, B. D., Chen, J. C., Johnson, E. E., and Johnson, A. E. (1997). The aqueous pore through the translocon has a diameter of 40-60 A during cotranslational protein translocation at the ER membrane. *Cell* **89,** 535-44.

Hammond, C., Braakman, I., and Helenius, A. (1994). Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc Natl Acad Sci U S A* **91,** 913-7.

Hammond, C., and Helenius, A. (1995a). Quality control in the secretory pathway. *Curr Opin Cell Biol* **7,** 523-9.

Hammond, C., and Helenius, A. (1995b). Quality control in the secretory pathway. *Curr Opin Cell Biol* **7,** 523-9.

Hampton, R. Y., and Bhakta, H. (1997). Ubiquitin-mediated regulation of 3-hydroxy-3-methylglutaryl-CoA reductase. *Proc Natl Acad Sci U S A* **94,** 12944-8.

Hampton, R. Y., Gardner, R. G., and Rine, J. (1996). Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3- methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol Biol Cell* **7,** 2029-44.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. *J Mol Biol* **166,** 557-80.

Hanein, D., Matlack, K. E., Jungnickel, B., Plath, K., Kalies, K. U., Miller, K. R., Rapoport, T. A., and Akey, C. W. (1996). Oligomeric rings of the Sec61p complex induced by ligands required for protein translocation. *Cell* **87,** 721-32.

Hartmann-Petersen, R., and Gordon, C. (2004). Proteins interacting with the 26S proteasome. *Cell Mol Life Sci* **61,** 1589-95.

Heessen, S., Dantuma, N. P., Tessarz, P., Jellne, M., and Masucci, M. G. (2003). Inhibition of ubiquitin/proteasome-dependent proteolysis in Saccharomyces cerevisiae by a Gly-Ala repeat. *FEBS Lett* **555,** 397-404.

Heessen, S., Leonchiks, A., Issaeva, N., Sharipo, A., Selivanova, G., Masucci, M. G., and Dantuma, N. P. (2002). Functional p53 chimeras containing the Epstein-Barr virus Gly-Ala repeat are protected from Mdm2- and HPV-E6-induced proteolysis. *Proc Natl Acad Sci U S A* **99,** 1532-7.

Heinemeyer, W., Fischer, M., Krimmer, T., Stachon, U., and Wolf, D. H. (1997). The active sites of the eukaryotic 20 S proteasome and their involvement in subunit precursor processing. *J Biol Chem* **272,** 25200-9.

Helenius, A., and Aebi, M. (2001). Intracellular functions of N-linked glycans. *Science* **291,** 2364-9.

Helenius, A., and Aebi, M. (2004). Roles of N-Linked Glycans in the Endoplasmic Reticulum. *Annu Rev Biochem* **73,** 1019-1049.

Helenius, A., Trombetta, E. S., Hebert, D. N., and Simons, J. F. (1997). Calnexin, calreticulin and the folding of glycoproteins. *Trends Cell Biol* **7,** 193-200.

Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996). ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science* **273,** 1725-8.

Hitt, R. (2003). Proteinqualitaetskontrolle im endoplasmatischen Retikulum: Identifizierung und Charakterisierung von Komponenten des Abbau missgefalteter Proteine. *In* "Insititut fuer Biochemie". Universitaet Stuttgart, Stuttgart.

Hitt, R., and Wolf, D. H. (2004). Der1p, a protein required for degradation of malfolded soluble proteins of the endoplasmic reticulum: topology and Der1-like proteins. *FEMS Yeast Res* **4,** 721-9.

Hitt, R., Wolf, D.H. (2004). DER7, encoding alpha-glucosidase I is essential for degradation of malfolded glycoproteins of the endoplasmic reticulum. *FEMSYR* **in press**.

Hoyt, M. A., Zhang, M., and Coffino, P. (2003). Ubiquitin-independent mechanisms of mouse ornithine decarboxylase degradation are conserved between mammalian and fungal cells. *J Biol Chem* **278,** 12135-43.

Huppa, J. B., and Ploegh, H. L. (1997). The alpha chain of the T cell antigen receptor is degraded in the cytosol. *Immunity* **7,** 113-22.

Huyer, G., Piluek, W. F., Fansler, Z., Kreft, S. G., Hochstrasser, M., Brodsky, J. L., and Michaelis, S. (2004). Distinct machinery is required in Saccharomyces cerevisiae for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein. *J Biol Chem* **279,** 38369-78.

Huyton, T., Pye, V. E., Briggs, L. C., Flynn, T. C., Beuron, F., Kondo, H., Ma, J., Zhang, X., and Freemont, P. S. (2003). The crystal structure of murine p97/VCP at 3.6A. *J Struct Biol* **144,** 337-48.

Jakob, C. A., Bodmer, D., Spirig, U., Battig, P., Marcil, A., Dignard, D., Bergeron, J. J., Thomas, D. Y., and Aebi, M. (2001). Htm1p, a mannosidase-like protein, is involved in glycoprotein degradation in yeast. *EMBO Rep* **2,** 423-430.

Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D. H., and Sommer, T. (2002). Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nat Cell Biol* **4,** 134-9.

Jensen, T. J., Loo, M. A., Pind, S., Williams, D. B., Goldberg, A. L., and Riordan, J. R. (1995). Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell* **83,** 129-35.

Johnson, A. E., and Haigh, N. G. (2000). The ER translocon and retrotranslocation: is the shift into reverse manual or automatic? *Cell* **102,** 709-12.

Johnson, A. E., and van Waes, M. A. (1999). The translocon: a dynamic gateway at the ER membrane. *Annu Rev Cell Dev Biol* **15,** 799-842.

Kalies, K. U., Allan, S., Sergeyenko, T., Kroger, H., and Romisch, K. (2005). The protein translocation channel binds proteasomes to the endoplasmic reticulum membrane. *Embo J* **24,** 2284-93.

Katiyar, S., Li, G., and Lennarz, W. J. (2004). A complex between peptide:N-glycanase and two proteasome-linked proteins suggests a mechanism for the degradation of misfolded glycoproteins. *Proc Natl Acad Sci U S A* **101,** 13774-9.

Kern, G., Schulke, N., Schmid, F. X., and Jaenicke, R. (1992). Stability, quaternary structure, and folding of internal, external, and core-glycosylated invertase from yeast. *Protein Sci* **1,** 120-31.

Kikkert, M., Hassink, G., Barel, M., Hirsch, C., van der Wal, F. J., and Wiertz, E. (2001). Ubiquitination is essential for human cytomegalovirus US11-mediated dislocation of MHC class I molecules from the endoplasmic reticulum to the cytosol. *Biochem J* **358,** 369-77.

Kiser, G. L., Gentzsch, M., Kloser, A. K., Balzi, E., Wolf, D. H., Goffeau, A., and Riordan, J. R. (2001). Expression and degradation of the cystic fibrosis transmembrane conductance regulator in Saccharomyces cerevisiae. *Arch Biochem Biophys* **390,** 195-205.

Knop, M., Finger, A., Braun, T., Hellmuth, K., and Wolf, D. H. (1996a). Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. *Embo J* **15,** 753-63.

Knop, M., Hauser, N., and Wolf, D. H. (1996b). N-Glycosylation affects endoplasmic reticulum degradation of a mutated derivative of carboxypeptidase yscY in yeast. *Yeast* **12,** 1229-38.

Kondo, H., Rabouille, C., Newman, R., Levine, T. P., Pappin, D., Freemont, P., and Warren, G. (1997). p47 is a cofactor for p97-mediated membrane fusion. *Nature* **388,** 75-8.

Kostova, Z., and Wolf, D. H. (2002). Protein Quality Control in the Export Pathway: The Endoplasmic Retikulum and its Cytoplasmic Proteasome Connection. *In* "Protein Targeting, Transport and Translocation" (R. E. Dalbey and G. von Heijne, Eds.), pp. 180-213. Academic Press, London-New York.

Kostova, Z., and Wolf, D. H. (2003). For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin-proteasome connection. *Embo J* **22,** 2309-17.

Kowalski, J. M., Parekh, R. N., Mao, J., and Wittrup, K. D. (1998a). Protein folding stability can determine the efficiency of escape from endoplasmic reticulum quality control. *J Biol Chem* **273,** 19453-8.

Kowalski, J. M., Parekh, R. N., and Wittrup, K. D. (1998b). Secretion efficiency in Saccharomyces cerevisiae of bovine pancreatic trypsin inhibitor mutants lacking disulfide bonds is correlated with thermodynamic stability. *Biochemistry* **37,** 1264-73.

Lämmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of *Bacteriophage T4*. *Nature* **227,** 680-685.

Levitskaya, J., Coram, M., Levitsky, V., Imreh, S., Steigerwald-Mullen, P. M., Klein, G., Kurilla, M. G., and Masucci, M. G. (1995). Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature* **375,** 685-8.

Levitskaya, J., Sharipo, A., Leonchiks, A., Ciechanover, A., and Masucci, M. G. (1997). Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. *Proc Natl Acad Sci U S A* **94,** 12616-21.

Lilley, B. N., and Ploegh, H. L. (2004). A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* **429,** 834-40.

Loayza, D., Tam, A., Schmidt, W. K., and Michaelis, S. (1998). Ste6p mutants defective in exit from the endoplasmic reticulum (ER) reveal aspects of an ER quality control pathway in Saccharomyces cerevisiae. *Mol Biol Cell* **9,** 2767-84.

Medicherla, B., Kostova, Z., Schaefer, A., and Wolf, D. H. (2004). A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. *EMBO Rep* **5,** 692-7.

Meigs, T. E., and Simoni, R. D. (1997). Farnesol as a regulator of HMG-CoA reductase degradation: characterization and role of farnesyl pyrophosphatase. *Arch Biochem Biophys* **345,** 1-9.

Menetret, J. F., Neuhof, A., Morgan, D. G., Plath, K., Radermacher, M., Rapoport, T. A., and Akey, C. W. (2000). The structure of ribosome-channel complexes engaged in protein translocation. *Mol Cell* **6,** 1219-32.

Meyer, H. H., Shorter, J. G., Seemann, J., Pappin, D., and Warren, G. (2000). A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. *Embo J* **19,** 2181-92.

Miller, D. M., Delgado, R., Chirgwin, J. M., Hardies, S. C., and Horowitz, P. M. (1991). Expression of cloned bovine adrenal rhodanese. *J Biol Chem* **266,** 4686-91.

Mingarro, I., Nilsson, I., Whitley, P., and von Heijne, G. (2000). Different conformations of nascent polypeptides during translocation across the ER membrane. *BMC Cell Biol* **1,** 3.

Misaghi, S., Pacold, M. E., Blom, D., Ploegh, H. L., and Korbel, G. A. (2004). Using a small molecule inhibitor of peptide: N-glycanase to probe its role in glycoprotein turnover. *Chem Biol* **11,** 1677-87.

Morgan, D. G., Menetret, J. F., Radermacher, M., Neuhof, A., Akey, I. V., Rapoport, T. A., and Akey, C. W. (2000). A comparison of the yeast and rabbit 80 S ribosome reveals the topology of the nascent chain exit tunnel, inter-subunit bridges and mammalian rRNA expansion segments. *J Mol Biol* **301,** 301-21.

Musch, A., Wiedmann, M., and Rapoport, T. A. (1992). Yeast Sec proteins interact with polypeptides traversing the endoplasmic reticulum membrane. *Cell* **69,** 343-52.

Nakatsukasa, K., Nishikawa, S., Hosokawa, N., Nagata, K., and Endo, T. (2001). Mnl1p, an alpha -mannosidase-like protein in yeast Saccharomyces cerevisiae, is required for endoplasmic reticulum-associated degradation of glycoproteins. *J Biol Chem* **276,** 8635-8.

Newman, R. H., Whitehead, P., Lally, J., Coffer, A., and Freemont, P. (1996). 20S human proteasomes bind with a specific orientation to lipid monolayers in vitro. *Biochim Biophys Acta* **1281,** 111-6.

Nock, S., Gonzalez, T. N., Sidrauski, C., Niwa, M., and Walter, P. (2001). Purification and activity assays of the catalytic domains of the kinase/endoribonuclease Ire1p from Saccharomyces cerevisiae. *Methods Enzymol* **342,** 3-10.

Oliver Neuber, e. J., Corinna Volkwein, Jan Walter and Thomas Sommer. (2005). Ubx2 links the Cdc48 complex to ER-associated protein degradation. *nature cell biology* **7,** 993-998.

Orr-Weaver, T. L., Szostak, J. W., and Rothstein, R. J. (1981). Yeast transformation: a model system for the study of recombination. *Proc Natl Acad Sci U S A* **78,** 6354-8.

Orr-Weaver, T. L., Szostak, J. W., and Rothstein, R. J. (1983). Genetic applications of yeast transformation with linear and gapped plasmids. *Methods Enzymol* **101,** 228-45.

Palmer, A., Rivett, A. J., Thomson, S., Hendil, K. B., Butcher, G. W., Fuertes, G., and Knecht, E. (1996). Subpopulations of proteasomes in rat liver nuclei, microsomes and cytosol. *Biochem J* **316 (Pt 2),** 401-7.

Panzner, S., Dreier, L., Hartmann, E., Kostka, S., and Rapoport, T. A. (1995). Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. *Cell* **81,** 561-70.

Parekh, R., Forrester, K., and Wittrup, D. (1995). Multicopy overexpression of bovine pancreatic trypsin inhibitor saturates the protein folding and secretory capacity of Saccharomyces cerevisiae. *Protein Expr Purif* **6,** 537-45.

Pilon, M., Schekman, R., and Romisch, K. (1997). Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. *Embo J* **16,** 4540-8.

Plemper, R. K., Bohmler, S., Bordallo, J., Sommer, T., and Wolf, D. H. (1997). Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature* **388,** 891-5.

Plemper, R. K., Bordallo, J., Deak, P. M., Taxis, C., Hitt, R., and Wolf, D. H. (1999a). Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. *J Cell Sci* **112,** 4123-34.

Plemper, R. K., Deak, P. M., Otto, R. T., and Wolf, D. H. (1999b). Re-entering the translocon from the lumenal side of the endoplasmic reticulum. Studies on mutated carboxypeptidase yscY species. *FEBS Lett* **443,** 241-5.

Plemper, R. K., Egner, R., Kuchler, K., and Wolf, D. H. (1998). Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdr5 proceeds in a concerted action of Sec61 and the proteasome. *J Biol Chem* **273,** 32848-56.

Ploegman, J. H., Drent, G., Kalk, K. H., Hol, W. G., Heinrikson, R. L., Keim, P., Weng, L., and Russell, J. (1978). The covalent and tertiary structure of bovine liver rhodanese. *Nature* **273,** 124-9.

Prinz, W. A., Grzyb, L., Veenhuis, M., Kahana, J. A., Silver, P. A., and Rapoport, T. A. (2000). Mutants affecting the structure of the cortical endoplasmic reticulum in Saccharomyces cerevisiae. *J Cell Biol* **150,** 461-74.

Rabinovich, E., Kerem, A., Frohlich, K. U., Diamant, N., and Bar-Nun, S. (2002). AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. *Mol Cell Biol* **22,** 626-34.

Rabouille, C., Kondo, H., Newman, R., Hui, N., Freemont, P., and Warren, G. (1998). Syntaxin 5 is a common component of the NSF- and p97-mediated reassembly pathways of Golgi cisternae from mitotic Golgi fragments in vitro. *Cell* **92,** 603-10.

Rapoport, T. A. (1992). Transport of proteins across the endoplasmic reticulum membrane. *Science* **258,** 931-6.

Rapoport, T. A., Jungnickel, B., and Kutay, U. (1996). Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu Rev Biochem* **65,** 271-303.

Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C., and Jentsch, S. (2005). A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell* **120,** 73-84.

Rivett, A. J., and Knecht, E. (1993). Proteasome location. *Curr.Biol.* **3,** 127-129.

Roberts, B. J. (1997). Evidence of proteasome-mediated cytochrome P-450 degradation. *J Biol Chem* **272,** 9771-8.

Romisch, K. (1999). Surfing the Sec61 channel: bidirectional protein translocation across the ER membrane. *J Cell Sci* **112 (Pt 23),** 4185-91.

Romisch, K. (2005). Endoplasmic Reticulum-Associated Degradation. *Annu Rev Cell Dev Biol*.

Rose, M. D., Misra, L. M., and Vogel, J. P. (1989). KAR2, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. *Cell* **57,** 1211-21.

Rouiller, I., Butel, V. M., Latterich, M., Milligan, R. A., and Wilson-Kubalek, E. M. (2000). A major conformational change in p97 AAA ATPase upon ATP binding. *Mol Cell* **6,** 1485-90.

Saito, Y., Yamanushi, T., Oka, T., and Nakano, A. (1999). Identification of SEC12, SED4, truncated SEC16, and EKS1/HRD3 as multicopy suppressors of ts mutants of Sar1 GTPase. *J Biochem (Tokyo)* **125,** 130-7.

Schagger, H. (2001). Blue-native gels to isolate protein complexes from mitochondria. *Methods Cell Biol* **65,** 231-244.

Schagger, H., Cramer, W. A., and von Jagow, G. (1994). Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal Biochem* **217,** 220-30.

Schagger, H., and von Jagow, G. (1991). Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal Biochem* **199,** 223-31.

Schlenstedt, G., Zimmermann, M., and Zimmermann, R. (1994). A stably folded presecretory protein associates with and upon unfolding translocates across the membrane of mammalian microsomes. *FEBS Lett* **340,** 139-44.

Schuberth, C., and Buchberger, A. (2005). Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. *nature cell biology* **7,** 999-1006.

Schuberth, C., Richly, H., Rumpf, S., and Buchberger, A. (2004). Shp1 and Ubx2 are adaptors of Cdc48 involved in ubiquitin-dependent protein degradation. *EMBO Rep* **5,** 818-24.

Shamu, C. E., Flierman, D., Ploegh, H. L., Rapoport, T. A., and Chau, V. (2001). Polyubiquitination is required for US11-dependent movement of MHC class I heavy chain from endoplasmic reticulum into cytosol. *Mol Biol Cell* **12,** 2546-55.

Sidrauski, C., and Walter, P. (1997). The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response. *Cell* **90,** 1031-9.

Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122,** 19-27.

Sommer, T., and Wolf, D. H. (1997). Endoplasmic reticulum degradation: reverse protein flow of no return. *Faseb J* **11,** 1227-33.

Soni, R., Carmichael, J. P., and Murray, J. A. (1993). Parameters affecting lithium acetate-mediated transformation of Saccharomyces cerevisiae and development of a rapid and simplified procedure. *Curr Genet* **24,** 455-9.

Spiro, R. G. (2004). Role of N-linked polymannose oligosaccharides in targeting glycoproteins for endoplasmic reticulum-associated degradation. *Cell Mol Life Sci* **61,** 1025-41.

Suzuki, T., Park, H., Hollingsworth, N. M., Sternglanz, R., and Lennarz, W. J. (2000). PNG1, a yeast gene encoding a highly conserved peptide:N-glycanase. *J Cell Biol* **149,** 1039-52.

Suzuki, T., Park, H., Kwofie, M. A., and Lennarz, W. J. (2001). Rad23 provides a link between the Png1 deglycosylating enzyme and the 26S proteasome in yeast. *J Biol Chem* **276,** published online on March 20.

Swanson, R., Locher, M., and Hochstrasser, M. (2001). A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. *Genes Dev* **15,** 2660-74.

Taxis, C., Hitt, R., Park, S. H., Deak, P. M., Kostova, Z., and Wolf, D. H. (2003). Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. *J Biol Chem* **278,** 35903-13.

Thillet, J., Absil, J., Stone, S. R., and Pictet, R. (1988). Site-directed mutagenesis of mouse dihydrofolate reductase. Mutants with increased resistance to methotrexate and trimethoprim. *J Biol Chem* **263,** 12500-8.

Tirosh, B., Furman, M. H., Tortorella, D., and Ploegh, H. L. (2003). Protein unfolding is not a prerequisite for endoplasmic reticulum-to-cytosol dislocation. *J Biol Chem* **278,** 6664-72.

Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and Walter, P. (2000a). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101,** 249-58.

Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and Walter, P. (2000b). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101,** 249-58.

Wach, A., Brachat, A., Alberti-Segui, C., Rebischung, C., and Philippsen, P. (1997). Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. *Yeast* **13,** 1065-1075.

Walter, J., Urban, J., Volkwein, C., and Sommer, T. (2001). Sec61p-independent degradation of the tail-anchored ER membrane protein Ubc6p. *Embo J* **20,** 3124-31.

Ward, C. L., Omura, S., and Kopito, R. R. (1995). Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* **83,** 121-7.

Werner, E. D., Brodsky, J. L., and McCracken, A. A. (1996). Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. *Proc Natl Acad Sci U S A* **93,** 13797-801.

Wiertz, E. J., Jones, T. R., Sun, L., Bogyo, M., Geuze, H. J., and Ploegh, H. L. (1996a). The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* **84,** 769-79.

Wiertz, E. J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T. R., Rapoport, T. A., and Ploegh, H. L. (1996b). Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* **384,** 432-8.

Wolf, D. H. (2004). Ubiquitin-proteasome systemFrom lysosome to proteasome: the power of yeast in the dissection of proteinase function in cellular regulation and waste disposal. *Cell Mol Life Sci* **61,** 1601-14.

Wolf, D. H., and Hilt, W. (2004). The proteasome: a proteolytic nanomachine of cell regulation and waste disposal. *Biochim Biophys Acta* **1695,** 19-31.

Wolf, D. H., and Schafer, A. (2005). CPY* and the power of yeast genetics in the elucidation of quality control and associated protein degradation of the endoplasmic reticulum. *Curr Top Microbiol Immunol* **300,** 41-56.

Yang, M., Omura, S., Bonifacino, J. S., and Weissman, A. M. (1998). Novel aspects of degradation of T cell receptor subunits from the endoplasmic reticulum (ER) in T cells: importance of oligosaccharide processing, ubiquitination, and proteasome-dependent removal from ER membranes. *J Exp Med* **187,** 835-46.

Ye, Y., Meyer, H. H., and Rapoport, T. A. (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* **414,** 652-6.

Ye, Y., Meyer, H. H., and Rapoport, T. A. (2003). Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. *J Cell Biol* **162,** 71-84.

Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T. A. (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* **429,** 841-7.

Yu, H., Kaung, G., Kobayashi, S., and Kopito, R. R. (1997). Cytosolic degradation of T-cell receptor alpha chains by the proteasome. *J Biol Chem* **272,** 20800-4.

Zhang, M., MacDonald, A. I., Hoyt, M. A., and Coffino, P. (2004). Proteasomes begin ornithine decarboxylase digestion at the C terminus. *J Biol Chem* **279,** 20959-65.

Zhang, X., Shaw, A., Bates, P. A., Newman, R. H., Gowen, B., Orlova, E., Gorman, M. A., Kondo, H., Dokurno, P., Lally, J., Leonard, G., Meyer, H., van Heel, M., and Freemont, P. S. (2000). Structure of the AAA ATPase p97. *Mol Cell* **6,** 1473-84.

Zhong, X., Shen, Y., Ballar, P., Apostolou, A., Agami, R., and Fang, S. (2004). AAA ATPase p97/valosin-containing protein interacts with gp78, a ubiquitin ligase for endoplasmic reticulum-associated degradation. *J Biol Chem* **279,** 45676-84.

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Erklärung

Hiermit versichere ich, dass ich diese Arbeit selbst verfasst und dabei keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

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Li Xiao