Molecular Chaperones in Protein Quality Control: From Recognition to Degradation

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Abbreviations

△ss	Deletion of signal sequence
µl	Microliter
AAA	ATPase associated with a variety of cellular activities
Amp	Ampicillin
ATP	Adenosine 5´-triphosphate
ATPase	Adenosintriphosphatase
BAG	Bcl-2-associated athanogene
BSA	Bovine serum albumine
CHX	Cycloheximide
CG*	Mutated Carboxypeptidase Y-GFP
CM	Complete minimum dropout medium
CPY	Carboxypeptidase yscY
CPY*	Mutated form of CPY (allele *prc1-1*)
CT*	Mutated Carboxypeptidase Y-Transmembrane domain
CTG*	Mutated Carboxypeptidase Y-Transmembrane domain-GFP
Da	Dalton
ddH2O	Double deionised water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	D,L Dithiothreitol
E. coli	*Escherichia coli*

Abbreviations

ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
ER	Endoplasmic reticulum
ERAD	ER associated degradation
Fig	Figure
g	Gram
hr	Hour
HA	Haemaglutinin
Hect	homologous to E6-AP c-terminus
HRPO	Horse radish peroxidase
kb	Kilobase pairs
kDa	Kilodalton
l	Liter
IB	Immuno Blot
IP	Immuno Precipitation
LB	Luria Broth
M	Molar
mg	Milligram
min	Minute
ml	Millilitres
mM	Millimolar
OD_{600}	Optical density at 600 nm
ODC	Ornithine decarboxylase
ORF	Open reading frame

Abbreviations

PBS	Phosphate buffer saline
PEG	Polyethyleneglycol
rpm	Rotations per minute
RT	Room temperature
SDS	Sodium-Dodecyl-Sulfate
PAGE	Polyacrylamide Gel Electrophoresis
T4-ligase	Bacteriophage T4 Ligase
TAE	Tris acetate EDTA
TCA	Trichloroaceticacid
TE	Tris EDTA
TEMED	Tetramethylethyldiamine
Tris	Tris(hydroxymethyl)aminomethane
TritonX 100	Alkylpehnylpolyethylenglycol
Tween 20	Polyoxyethylensorbitolmonolaurate
Ub	Ubiquitin
V	Volts
v/v	Volume/ Volume
w/v	Weight/ Volume

Zusammenfassung

Das endoplasmatische Retikulum (ER) beherbergt ein Proteinqualitätskontrollsystem, durch das die Proteinfaltung im ER kontrolliert wird. Der Abbau von falschgefalteten Proteinen ist eine wichtige Funktion dieser Proteinqualitätskontrolle. Aus früheren Untersuchungen mit verschiedenen löslichen und membrandurchspannenden Substraten des ERAD (ER-assoziierte Degradation) Signalweges ist bekannt, dass die einzelnen Substrate einen unterschiedlichen Aufbau der ER Degradationsmaschinerie benötigen. Um die Grundlagen dieser Unterschiede zu enträtseln, wurden zwei Typ I membrandurchspannende ERAD Substrate erzeugt, welche die fehlgefaltete Carboxypeptidase yscY (CPY*) als ER lumenales ERAD Erkennungsmotiv besitzen. Das Substrat CT* (CPY*-TM) besitzt keine zytoplasmatische Domäne mehr, wogegen das Substrat CTG* das grünfluoreszierende Protein „GFP“ im Zytosol präsentiert. Zusammen mit dem löslichen CPY* stellen alle drei Substrate hinsichtlich der Topologie unterschiedliche fehlgefaltete Proteine dar, welche über den ERAD Signalweg abgebaut werden.

Diese Studie zeigt, dass der Abbau dieser 3 Proteine abhängig vom Ubiquitin-Proteasom System, einschließlich des Ubiquitin-Ligase Komplexes Der3/Hrd1p-Hrd3p, der Ubiquitin konjugierenden Enzymen Ubc1p und Ubc7p, als auch des AAA-ATPase Komplexes Cdc48-Ufd1-Npl4 und des 26S Proteasomes erfolgt. Im Gegensatz zum löslichen CPY*, benötigen die membrangebunden Proteine CT* und CTG* kein Kar2p (BiP) und Der1p, zwei ER lokalisierte Proteine. Stattdessen benötigt CTG* für seinen Abbau die zytosolischen Chaperone Hsp70p, Hsp40p und Hsp104p. Noch vor der Aktivität des Cdc48p-Ufd1p-Npl4p Komplexes benötigt polyubiquitiniertes CTG* für

seine Dislokation die Chaperonaktivität von Ssa1p. Die Entdeckung, dass die Funktion von Ssa1p nicht auf ERAD Substrate limitiert ist, zeigt die allgemeine Bedeutung dieses Chaperons für den Abbau von unerwünschten Proteinen durch das Ubiquitin Proteasom system im zellulären Kontext.

Über die grundlegenden Mechanismen der Qualitätskontrolle und den Abbau von Proteinen im Zytoplasma ist bisher wenig bekannt. Daher untersuchten wir die Beteiligung von zytoplasmatischen Faktoren, die für den Abbau von ΔssCPY* und ΔssCPY*-GFP benötigt werden. ΔssCPY* und ΔssCPY*-GFP sind zwei ER-Import-defiziente, mutierte Versionen der Carboxypepdidase Y. Zusätzlich wurden die beteiligten Komponenten für den Abbau des entsprechenden Wildtypenzyms (ΔssCPY) untersucht, welches durch die Entfernung seiner ER-Signalsequenz ebenfalls importdefizient gemacht wurde. Allen genannten Proteinen ist gemeinsam, dass sie schnell durch das Ubiquitin Proteasom System abgebaut werden. Ihr Abbau erfordert die Ubiquitin konjugierenden Enzyme Ubc4p und Ubc5p, die zytosolische Hsp70 Ssa Chaperon Maschinerie und das Hsp40 Cochaperon Ydj1p. Hsp90 Chaperone sind am Abbauprozess nicht beteiligt.

Die Degradation eines GFP Fusionsproteins (GFP-cODC), das die C-terminalen 37 Aminosäuren der Ornithindecarboxylase (cODC) besitzt, wodurch dieses Enzym zum Proteasom geleitet wird, ist unabhängig von der Ssa1p Funktion. Die Fusion von ΔssCPY* und GFP-cODC zu ΔssCPY-GFP-cODC löst wiederum die Abhängigkeit von Ssa1p Chaperonen für den Abbau auf. In diesem Zusammenhang prüften wir, ob Mutationen in dem Ubiquitin Modifikationsbereich von *SSA1* einen Einfluss auf die Chaperonaktivität beim Abbau von ΔssCG* haben. Jedoch konnte in den drei mutierten

SSA1 Allelen, *SSA1*^{K521R}, *SSA1*^{K536R} und *SSA1*$^{K521R- K536R}$ keine Veränderung des Abbaus von ΔssCG* gefunden werden.

Offenbar schreiben die fehlgefalteten Proteindomänen den Weg zur spezifischen Proteinentfernung vor. Diese Daten und unsere weiteren Ergebnisse liefern Hinweise dafür, dass die Ssa1p-Ydj1p Maschinerie fehlgefaltete Proteindomänen erkennt, fehlgefaltete Proteine löslich hält, bereits präzipitiertes Proteinmaterial wieder in Lösung bringt und fehlgefaltete, ubiquitinierte Proteine zum Proteasom eskortiert und dort zu deren Degradation abliefert.

Summary

The endoplasmic reticulum (ER) harbours a protein quality control system which monitors protein folding in the ER. Elimination of misfolded proteins (called ERAD, ER-associated degradation) is an important function of this protein quality control process. Earlier studies with various soluble and transmembrane (TM) ERAD substrates revealed differences in the ER-degradation machinery used. To unravel the nature of these differences we generated two type I membrane ERAD substrates carrying misfolded carboxypeptidase yscY (CPY*) as the ER-luminal ERAD recognition motif. Whereas the first, CT* (CPY*-TM), has no cytoplasmic domain, the second, CTG*, carries the green fluorescent protein (GFP) present in the cytosol. Together with soluble CPY*, these three substrates represent topologically diverse misfolded proteins, degraded via ERAD. This study shows that degradation of all three proteins is dependent on the ubiquitin-proteasome-system involving the ubiquitin-protein-ligase complex Der3/Hrd1p-Hrd3p, the ubiquitin conjugating enzymes Ubc1p and Ubc7p as well as the AAA-ATPase complex Cdc48-Ufd1-Npl4 and the 26S proteasome. In contrast to soluble CPY*, degradation of the membrane proteins CT* and CTG* does not require the ER proteins Kar2p (BiP) and Der1p. Instead, CTG* degradation requires cytosolic Hsp70, Hsp40 and Hsp104p chaperones and the chaperone activity of Ssa1p is necessary for the dislocation of polyubiquitinated CTG* prior to the action of the Cdc48p-Ufd1p-Npl4p complex. The finding that the chaperone activity of Ssa1p is not limited to ERAD substrates indicates the general importance of this chaperone activity for the elimination of unwanted proteins by the ubiquitin-proteasome system in the cellular context.

Summary

The mechanism of protein quality control and elimination of misfolded proteins in the cytoplasm is poorly understood. We studied the involvement of cytoplasmic factors required for the degradation of two ER-import defective mutated derivatives of carboxypeptidase yscY, ΔssCPY* and ΔssCPY*-GFP, and also examined the requirements for degradation of the corresponding wild type enzyme made ER-import incompetent by removal of its signal sequence (ΔssCPY). All these protein species are rapidly degraded via the ubiquitin-proteasome system. Degradation requires the ubiquitin conjugating enzymes Ubc4p and Ubc5p, the cytoplasmic Hsp70 Ssa chaperone machinery and the Hsp40 co-chaperone Ydj1p. The Hsp90 chaperones are not involved in the degradation process. Elimination of a GFP fusion (GFP-cODC), containing the C-terminal 37 amino acids of ornithine decarboxylase (cODC) directing this enzyme to the proteasome, is independent of Ssa1p function. Fusion of ΔssCPY* to GFP-cODC to form ΔssCPY*-GFP-cODC re-initiates a dependency on the Ssa1p chaperone for degradation. Evidently, the misfolded protein domain dictates the route of protein elimination. These data and our further results give evidence that the Ssa1p-Ydj1p machinery recognizes misfolded protein domains, keeps misfolded proteins soluble, solubilizes precipitated protein material, and escorts and delivers ubiquitinated misfolded proteins to the proteasome for degradation.

1. Introduction

Newly synthesized proteins must fold into their native three dimensional structures and maintain this state throughout their lifetime. Molecular chaperones facilitate the initial folding of proteins to their native form, as well as the assembly of multi-protein complexes. Translocation of proteins into the endoplasmic reticulum or into mitochondria and their folding also relies on molecular chaperones associated with these cellular compartments (Caplan *et al.*, 1992; Parsell and Lindquist, 1993; Hartl, 1996; Frydman, 2001; Hartl and Hayer-Hartl, 2002; Anken *et al.*, 2005; Mayer and Bukau, 2005). Molecular chaperones are involved not only in the folding of proteins but also in their quality control. During the folding process the non-native polypeptide and folding intermediates often expose hydrophobic patches that are buried in the native conformation. Exposed hydrophobic patches are a signal of terminal misfolding of proteins. Failure of correct folding in polypeptides leads to their aggregation in the aqueous cellular environment. This may result in the formation of toxic protein precipitates, which are associated with severe diseases such as Alzheimer's disease, Parkinson-disease or Creutzfeldt-Jakob-disease in humans or bovine spongiform encephalopathy (BSE) in cattle (Kopito, 2000; Dobson, 2003; Goldberg, 2003; Barral *et al.*, 2004). Protein quality control by molecular chaperones includes recognition of misfolding, prevention of protein aggregation and facilitation of refolding of partially unfolded proteins (Goldberg, 2003; Kleizen and Braakman, 2004). This whole process is essential to all cells.

1.1 Endoplasmic reticulum quality control and degradation (ERQD)

The eukaryotic endoplasmic reticulum (ER) is the site of production of most membrane protein and lipids, and it is the entry point for proteins destined for secretion. Secretory proteins are synthesized on the ribosomes of the rough endoplasmic reticulum and translocated into the lumen of ER in an unfolded state, where they interact with molecular chaperones like the heat shock protein Hsp70 BiP (Kar2p in yeast) and its co-chaperone proteins and lectins like calnexin and calreticulin, and disulfide isomerases (PDI). These components of the ER facilitate protein folding, maturation and post-translocational modifications, which include glycosylation and disulfide bond formation (Kostova and Wolf, 2003). Before proteins are transported out of the ER they are subjected to a "quality control" process which assesses their state of folding and then, if they are correctly folded, allows them to leave for their site of action (Sommer and Wolf, 1997; Nishikawa *et al.*, 2001; Kostova *et al.*, 2003; Kleizen *et al.*, 2004; Schafer and Wolf, 2005, 2006). However, proteins that do not fold correctly can have several fates. Prolonged interactions of non-native proteins with ER chaperones leads to their retention in the ER for further cycles of quality control or they are retrotranslocated out of the ER and degraded in the cytosol by the ubiquitin-proteasome system (Heinemeyer *et al.*, 1991; Hiller *et al.*, 1996; Plemper *et al.*, 1997; Sommer *et al.*, 1997; Cyr *et al.*, 2002; Kostova *et al.*, 2003; Esser *et al.*, 2004; Hirsch *et al.*, 2004; Wolf and Hilt, 2004).

1.1.1 The unfolded protein response (UPR) and ER-associated degradation

When non-native or unfolded proteins accumulate in the ER, a process called the unfolded protein response (UPR) is initiated (Sidrauski and Walter, 1997). In yeast, the transmembrane kinase Ire1p, localized to the ER/Nuclear envelope, interacts with Kar2p through its lumenal domain. Both unfolded proteins and Ire1p compete for binding to Kar2p. However, a decrease in the concentration of free Kar2p due to an increase of unfolded proteins in ER lumen, leads to the dimerization of Ire1p, and a conformational change transmits a signal across the membrane and activates the cytoplasmic kinase activity. The kinase induces the transcription of gene products that facilitate the processing of aberrant proteins and attenuate protein translation, which reduces the amount of newly imported proteins into the ER (Travers *et al.*, 2000). If quality control by ER chaperones and folding enzymes is unsuccessful, aberrant proteins are eliminated from the ER by a mechanism termed ER-associated degradation (ERAD).

1.1.2 Roles of ER chaperones in ERAD

The lectin like chaperones, calnexin and calreticulin play an important role to recognize specific N-linked carbohydrate chains on glycoproteins for correct folding. N-Glycosylation of proteins in the ER is achieved by adding the core oligosaccharide $Glc_3Man_9GlcNAc_2$ to the Asn-X-Ser/Thr consensus sequence of proteins during their translocation across the ER membrane (Helenius *et al.*, 1997; Kostova *et al.*, 2003). As folding progresses, two of the outermost glucose residues of the N-linked glycan are trimmed by the glucosidases Ⅰ and Ⅱ (Knop *et al.*, 1996b). In mammalian cells, following quality control, the mono-glucosylated ($GlcMan_9GlcNAc_2$) proteins bound to calnexin and calreticulin are released as a protein with the carbohydrate structure

$Man_9GlcNAc_2$ (Helenius *et al.*, 1997; Kostova *et al.*, 2003; Helenius and Aebi, 2004). However if the glycoprotein is not properly folded, the *N*-glycan is re-glucosylated at the same position by a UDP-glucose glucosytransferase (UGGT). Therefore calnexin and calreticulin bind to and retain immature glycoproteins and facilitate their folding to prevent the release of aberrant proteins in the ER (Cabral *et al.*, 2001).

If glycoproteins cannot acquire correct folding within a given time, they are targeted for ERAD. Entry into the ERAD pathway requires the trimming of a single mannose by ER α1.2-mannosidase Ⅰ and subsequent recognition of the $Man_8GlcNAc_2$ moiety by EDEM (ER-degradation enhancing 1, 2-mannosidase like protein, Htm1p in yeast) (Hosokawa *et al.*, 2001; Jakob *et al.*, 2001; Oda *et al.*, 2003). In a recent studies, Yos9p has been elucidated as a new lectin like protein necessary for efficient degradation of glycosylated ERAD substrates (Buschhorn *et al.*, 2004; Szathmary *et al.*, 2005). One of the model proteins used to study ER quality control in yeast is a mutant form of carboxypeptidase yscY, (G255R), CPY* (Finger *et al.*, 1993; Knop *et al.*, 1993; Hiller *et al.*, 1996; Plemper *et al.*, 1997) This misfolded glycoprotein is translocated into the ER lumen and fully glycosylated but is not transported to the vacuole. Instead, it is retained in the ER and degraded by the ubiquitin-proteasome system. The degradation of misfolded, CPY* is dependent on both Htm1p and Yos9p but is independent of calnexin (Buschhorn *et al.*, 2004; Kostova and Wolf, 2005). Nevertheless, glycosylated proteins are not the only ones targeted for ERAD. The elimination of the non-glycosylated mutant Sec61 translocon protein Sec61-2p is totally independent of the lectin like chaperones of the ER (Biederer *et al.*, 1996; Buschhorn *et al.*, 2004).

In contrast to the lectin like chaperones, Hsp70 chaperones such as BiP (Kar2p in yeast) recognize exposed hydrophobic regions of polypeptides or unfolded proteins and

cycles of ATP binding and ADP release are coupled with the association and dissociation of the substrates (Plemper *et al.*, 1997; Brodsky *et al.*, 1999b; Ellgaard and Helenius, 2003; Kostova *et al.*, 2003; Sitia and Braakman, 2003). Hsp40 co-chaperones enhance the ATPase activity of Hsp70 and thus affect peptide capture by Hsp70. BiP, then, recognizes unfolded proteins and facilitates their folding and retains terminally misfolded proteins in a soluble conformation (Taxis *et al.*, 2003). In yeast, Kar2p is required for the degradation of soluble proteins such as mutant pro-α-factor and CPY* (Plemper *et al.*, 1997; Plemper *et al.*, 1998; Brodsky *et al.*, 1999b; Nishikawa *et al.*, 2001). However, it is not required for the degradation of misfolded membrane proteins. Pdr5*, a mutant form of the polytopic plasma membrane ABC transporter Pdr5p, and Sec61-2p are eliminated from the ER without the action of Kar2p. Another important role of Hsp70 chaperones is to prevent the aggregation of misfolded proteins prior to their retrotranslocation back to the cytosol. In the yeast ER, CPY* is maintained in a soluble form by the co-operation of Kar2p and its co-chaperone proteins, Jem1p and Scj1p. Both co-chaperones are members of the Hsp40 family which have an Hsp70-interacting J-domain. Scj1p and Jem1p could be necessary for triggering the release of Kar2p from such substrates in order for dislocation and degradation to take place. Yeast strains defective in Kar2p or co-chaperone function, show aggregation of misfolded proteins in the ER under restrictive temperatures and their degradation is severely impaired (Nishikawa *et al.*, 2001). Kar2p and its partner proteins may also play a role in the delivery of the soluble substrates to other, as yet unknown, components linking recognition to elimination (Nishikawa *et al.*, 2001; Kostova *et al.*, 2003).

The ER contains a large number of oxidoreductases that catalyze disulfide bond formation and isomerization for correct protein folding (Regeimbal and Bardwell, 2002).

PDI has also a function in ER quality control and serves to unfold cholera toxin during the retrotranslocation of the A1 chain (Gillece *et al.*, 1999; Tsai *et al.*, 2002). The PDI like protein Erp57p interacts with lectin like chaperones and allows the combined action of disulfide isomerization and enhances the efficiency of the folding process (Oliver *et al.*, 1999).

1.1.3 Retrotranslocation and degradation

The identification of the membrane-anchored ubiquitin-conjugating enzyme Ubc6p in yeast provided the first evidence that ubiquitination can occur at the cytosolic face of the ER (Sommer and Jentsch, 1993). It was initially believed that misfolded and aberrant proteins of ER were degraded by ER-resident proteinase and peptidases (Bonifacino and Klausner, 1994). However, the presence of unspecific proteinases in the ER was hard to reconcile with its primary function in folding and assembly. Another possibility was the involvement of the lysosome/vacuole in elimination of aberrant proteins from the ER. However, the findings that the misfolded vacuolar peptidase CPY* and the mutant secretory protein pro-α-factor were retained in the ER lumen and degraded by the proteasome system established the crucial concept of retrotranslocation (Hiller *et al.*, 1996; Werner *et al.*, 1996). All components of the ubiquitin-proteasome machinery identified so far and required for CPY* degradation are either cytosolic or located at the cytosolic side of the ER membrane. Although the link between recognition and delivery of misfolded proteins from Htm1p/EDEM, PDI or BiP to the retrotranslocation channel is still not clear, the Sec61 translocon is suggested to function as a component of forming the dislocation channel (Wiertz *et al.*, 1996; Pilon *et al.*, 1997; Plemper *et al.*, 1997; Plemper *et al.*, 1998). The Sec61 translocon is composed of

3 different subunits Sec61p, Sbh1p, and Sss1p (Sec61α, Sec61β, Sec61γ in mammals). Genetic studies in yeast revealed that a certain mutation within the yeast Sec61 complex caused a significant delay in the degradation of CPY* and other ERAD substrates, while protein import was affected only to a minor degree (Plemper *et al.*, 1997). Indeed, the ER membrane located ubiquitin ligase components Der3/Hrd1and Hrd3p genetically interacted with Sec61p (Plemper *et al.*, 1999). Alternative and/or auxiliary components of retrotranslocation channel could be Der1p, a ER membrane protein of unknown function (Knop *et al.*, 1996a; Knop *et al.*, 1996b), Kar2p, PDI, Htm1p, and Hrd3p, an ER membrane protein which functions together with the ubiquitin-protein ligase Der3p (Gardner *et al.*, 2000; Deak and Wolf, 2001). Considering co-operativity between recognition, dislocation and degradation, these components, probably, do not act independently of each other (Kostova *et al.*, 2003). Recent studies show that a fusion protein of MHC class Ⅰ heavy chain with a strongly folded GFP domain (EGFP-HCI) or dihydrofolate reductase (DHFR-HCI) might be retrotranslocated without unfolding of the tightly folded domains (Fiebiger *et al.*, 2002). The inner diameter of the translocon pore is considered to be 40-60 Å during protein translocation (Johnson and van Waes, 1999; Haigh and Johnson, 2002). Therefore it is suggested that in its active state the translocon could accommodate a folded GFP molecule with a length of 24 Å and diameter of 42 Å (Kostova *et al.*, 2003).

However dislocation might also take place through a channel completely different from the Sec61p channel. Even though it has been shown that mutations in Sec61 inhibit the degradation of ER lumenal proteins like CPY*, membrane proteins like MHC class I heavy chain and Pdr5* suggesting a role in retrotranslocation, degradation of Ubc6p is independent of Sec61 (Walter *et al.*, 2001). Ubc6p is thought to be inserted

into the ER membrane via its tail, independent of the Sec61 translocation pore. It is thought that this protein is also extracted from the ER membrane in the same way, independent of Sec61p for degradation by the proteasome.

1.1.4 Ubiquitination and targeting to the proteasome

Selective protein degradation via the ubiquitin-proteasome system is a major pathway conserved throughout eukaryotes (Hochstrasser, 1996; Varshavsky, 1997; Hershko and Ciechanover, 1998; Wolf *et al.*, 2004). Ubiquitin is a highly conserved protein of 76 amino acids present in all eukaryotes from yeast to mammals. The initial step of ubiquitination of proteins is mediated by three consecutive reactions: ubiquitin-activation via an E1 enzyme (Uba), ubiquitin-conjugation via E2 enzymes (Ubc's), and the action of ubiquitin protein ligases, E3's, which mediate the selection of substrate and facilitate its ubiquitination. In the past decade, genetic screening of various yeast mutants has identified a number of protein components of the ubiquitin machinery acting in ERAD. Ubiquitin-conjugation enzymes, Ubc6p, a tailed-anchored E2 and Ubc7p, a soluble cytoplasmic E2 which is recruited to the ER membrane via Cue1p (Biederer *et al.*, 1997) are the two best characterized components of the ubiquitin machinery in ERAD (Biederer *et al.*, 1996; Hiller *et al.*, 1996; Plemper *et al.*, 1998). Recently it was found that Ubc1p, a protein upregulated during UPR along with Ubc7p, also participates in ERAD (Friedlander *et al.*, 2000). The ER membrane located polytopic Der3p/Hrd1p is the RING-H2 finger domain E3 which functions together with the E2 Ubc7p in polyubiquitination of retrotranslocated ERAD substrates (Bays *et al.*, 2001; Deak *et al.*, 2001). Absence of Der3p leads to the accumulation of model ERAD substrates like CPY* due to failure in ubiquitination (Hiller *et al.*, 1996). The

function of Der3p as an E3 is dependent on its RING-H2 domain. Deletion of the RING-H2 domain or exchange of a single cysteine residue at position 399 against serine completely abolishes degradation of CPY* and Pdr5* (Bordallo and Wolf, 1999). A RING-H2 finger domain is defined by the position and distance between six cysteines and two histidines and is able to bind two zinc atoms. The RING-H2 finger is also essential for the interaction of Der3p with Ubc7p and the C399S mutant of RING-H2 finger is defective in binding Ubc7p. This finding shows that the RING-H2 domain of the ligase is crucial for recruitment of the E2 Ubc7p (Deak *et al.*, 2001). Many ubiquitin-protein ligases tend to self-ubiquitinate *in vitro* in the absence of other substrates (Lorick *et al.*, 1999) and the Der3p RING-H2 finger protein also shows *in vitro* self ubiquitination. Der3p also interacts with the ER membrane protein Hrd3p. In the absence of Hrd3p, Der3p is highly unstable, underlining the importance of Hrd3p in controlling the function of Der3p (Plemper *et al.*, 1999; Gardner *et al.*, 2000). In addition, Doa10p was identified as a second E3 involved in the degradation of misfolded and short-lived ER proteins (Swanson *et al.*, 2001). Doa10p is an ER membrane protein with a RING-HC domain. It was originally discovered in a screen for mutants involved in the degradation of the Matα2 repressor. Doa10p is involved in the degradation of the tail-anchored Ubc6p. Doa10p, along with Der3p, is also involved in the degradation of Pdr5* and ΔF508 CFTR expressed in yeast as well as of the mutated a-factor transporter protein, Ste6*p (Gnann *et al.*, 2004; Huyer *et al.*, 2004).

Since ERAD substrates are polyubiquitinated on the ER membrane, a delivery system must exist for degradation in the cytoplasm. Progressive polyubiquitination may serve as a ratcheting mechanism in moving the polypeptide from the retrotranslocation channel into the cytoplasm, where the long bulky polyubiquitin chains prevent the

polypeptide from slipping back into the ER (Kostova *et al.*, 2003). Recent studies indicate that the 26S proteasome is not directly involved in substrate extraction from the ER membrane. In mutants defective either in proteasome activity or in one of the 19S cap ATPase subunit of the proteasome, CPY* accumulates to a large extent in the cytoplasm (Jarosch *et al.*, 2002).

The trimeric AAA-ATPase Cdc48p (p97 in mammals)-Ufd1p-Npl4p complex is required for dislocation of ERAD substrates upstream of the proteasome. Mutants of Cdc48p and its partners Ufd1p and Npl4p show defects in the degradation of ERAD substrates like CPY* in yeast and MHC class I heavy chain in mammals. The polyubiquitinated substrates are still membrane associated (Ye *et al.*, 2001; Jarosch *et al.*, 2002; Rabinovich *et al.*, 2002). Recently it has been found that Cdc48p interacts with the ER membrane protein Ubx2p and Sph1p via their UBX (ubiquitin regulatory X) domain which is closely related to ubiquitin in structure even though its sequence similarity is low (Hartmann-Petersen *et al.*, 2004; Schuberth *et al.*, 2004; Neuber *et al.*, 2005). Indeed, recruitment of Cdc48p by Ubx2p is essential for turnover of both ER and non-ER substrates, whereas the UBA domain (ubiquitin-associated domain) of Ubx2p is specifically required for ERAD substrates (Neuber *et al.*, 2005). These findings suggest that the trimeric AAA-ATPase Cdc48p-Ufd1p-Npl4p complex may act as a motor that actively pulls polyubiquitinated substrates out of retrotranslocation channel by ubiquitin-binding proteins. Alternatively, it may mobilize already dislocated and polyubiquitinated substrates to the 26S proteasome (Medicherla *et al.*, 2004).

Recently two polyubiquitin chain binding proteins Dsk2p and Rad23p have been identified (Wilkinson *et al.*, 2001; Chen and Madura, 2002; Funakoshi *et al.*, 2002; Hartmann-Petersen *et al.*, 2003). Dsk2p and Rad23p are not 19S cap subunits of the

proteasome but possess an N-terminal ubiquitin like domain (UBL), which binds to a specific site on the 19S cap, and a C-terminal UBA domain, capable of binding polyubiquitin chains (Wilkinson *et al.*, 2001; Rao and Sastry, 2002; Hartmann-Petersen *et al.*, 2003). In the absence of Dsk2p and Rad23p, proteasomal degradation of ERAD substrates is significantly delayed and polyubiquitinated and completely dislocated substrates accumulate in the cytosol despite the presence of an active proteasome (Medicherla *et al.*, 2004). These characteristics suggest that substrates destined for degradation can bind to the UBA domain of Dsk2p and Rad23p through the polyubiquitin chain and, consequently, can be delivered to the proteasome by means of the UBL-19S cap interaction (Medicherla *et al.*, 2004; Richly *et al.*, 2005).

The emerging picture is, therefore, the following (Fig. 1): After polyubiquitination and partial dislocation of the substrate from the retrotranslocation channel, the ER associated Cdc48-Ufd1p-Npl4p complex binds the polyubiquitylated substrate in an ATP dependent manner, pulls it away from the ER membrane and hands it over to the proteasome via Dsk2p and Rad23p for degradation.

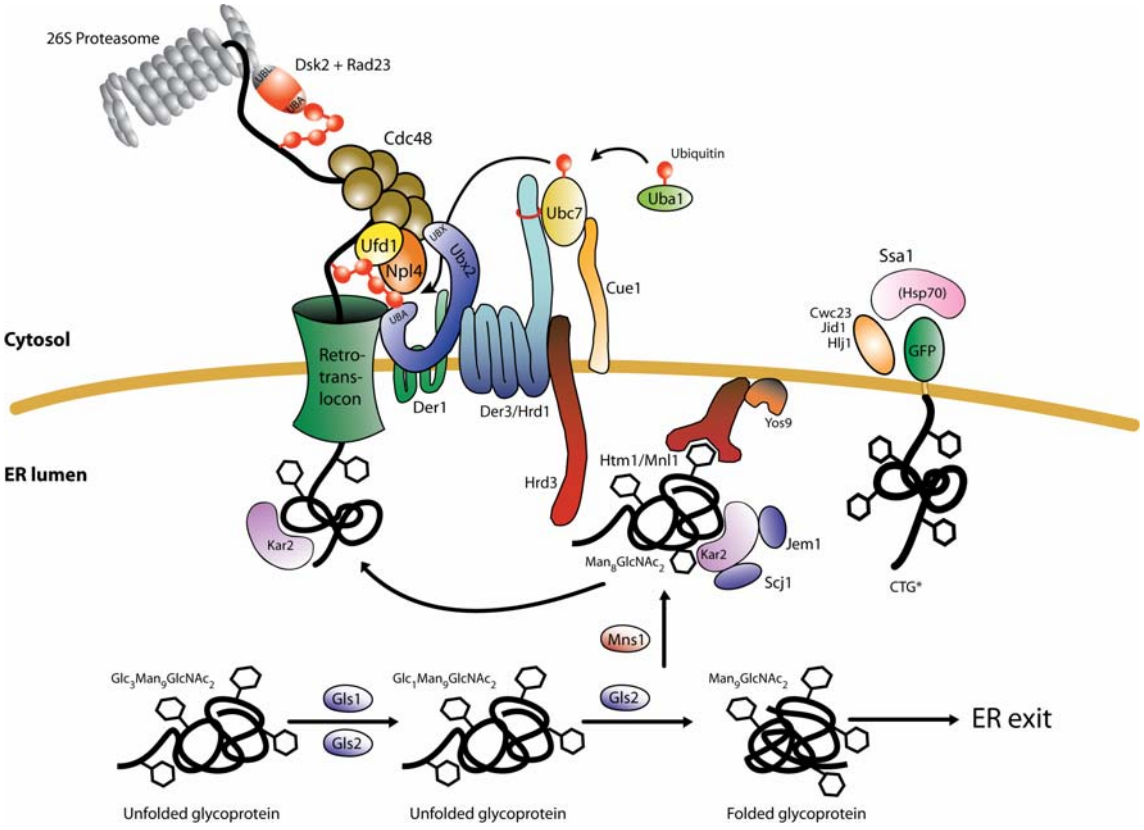

Figure 1. **Model of ER-associated protein degradation machinery in yeast** (Figure reproduced from Kostova and Wolf, 2003)

1.1.5 Degradation by the 26S proteasome

The 26S proteasome binds, unfolds and degrades the substrate proteins. With a few exceptions, like mutant α factor precursor (pαF) and ornithine decarboxylase (ODC), most proteins have to be polyubiquitinated prior to degradation (Werner *et al.*, 1996; Coffino, 2001; Wolf *et al.*, 2004). The 26S proteasome consists of the 20S proteolytic core particle (20S, CP) and the 19S regulatory complex (19S, RP) (Heinemeyer *et al.*, 1991; Voges *et al.*, 1999; Wolf *et al.*, 2004). The 20S proteolytic core particle is composed of four hetero-oligomeric rings consisting of two sets of seven different α-

and seven different β-type subunits in an $\alpha_7/\beta_7/\beta_7/\alpha_7$ architecture. The four rings enclose three inner compartments, two antechambers flanking one proteolytic chamber, which is build by the two β subunit rings in the middle. Three β-type subunits of each ring harbour the proteolytic active sites (Voges *et al.*, 1999; Wolf *et al.*, 2004). N-terminal stretches of the external α-subunits regulate the entry of substrates into the proteolytic core (Groll *et al.*, 1997). The 19S cap is involved in recognition, binding and unfolding of ubiquitinated proteins, and in the regulation of the opening of the 20S core. Access to the CP is restricted to unfolded proteins only. The RP can be found on both ends of the proteasome. Each unit consists of a base- and lid complex. The base complex contains 6 ATPases (Rpt1-6) of the "AAA" family (ATPases associated with a variety of cellular activities) and three non-ATPases (Rpn1, Rpn2, and Rpn10). The specific functions of the ATPase subunits in binding and unfolding are slowly emerging (Braun et al., 1999). Rpt5 binds ubiquitinated substrates (Lam *et al.*, 2002). Rpt2 is believed to control both substrate entry and product release from the 20S channel (Kohler *et al.*, 2001). Rpn1 interacts with Rad23 and Dsk2, two proteins having ubiquitin-like domains (UBL) and capable of binding and delivering ubiquitinated cargo to the proteasome. Recent findings suggest that Rpn10 contributes to the binding of ubiquitin chains as well (Elsasser and Finley, 2005) . The lid complex consists of 8 non-ATPase subunits (Rpn3-9, Rpn11-12). Rpn11 contains a highly conserved metallo isopeptidase motif and this activity is necessary for de-ubiquitination, and proteasomal proteolysis of substrates. It is currently believed that Rpn11 de-ubiquitinates the substrate after it has been threaded into the 20S channel, thereby resulting in an irreversible commitment to proteolysis. Failure to de-ubiquitinate probably causes a steric block for further insertion of the substrate into the proteolytic core (Verma and Deshaies, 2000).

Following release from the substrate, the polyubiquitin chain is hydrolyzed into single ubiquitin moieties, which can take part in a new round of protein degradation. After the polyubiquitin chain is cleaved off from the substrate protein, it is pushed through the 20S proteasome where it gets digested to small peptides (Fig. 2).

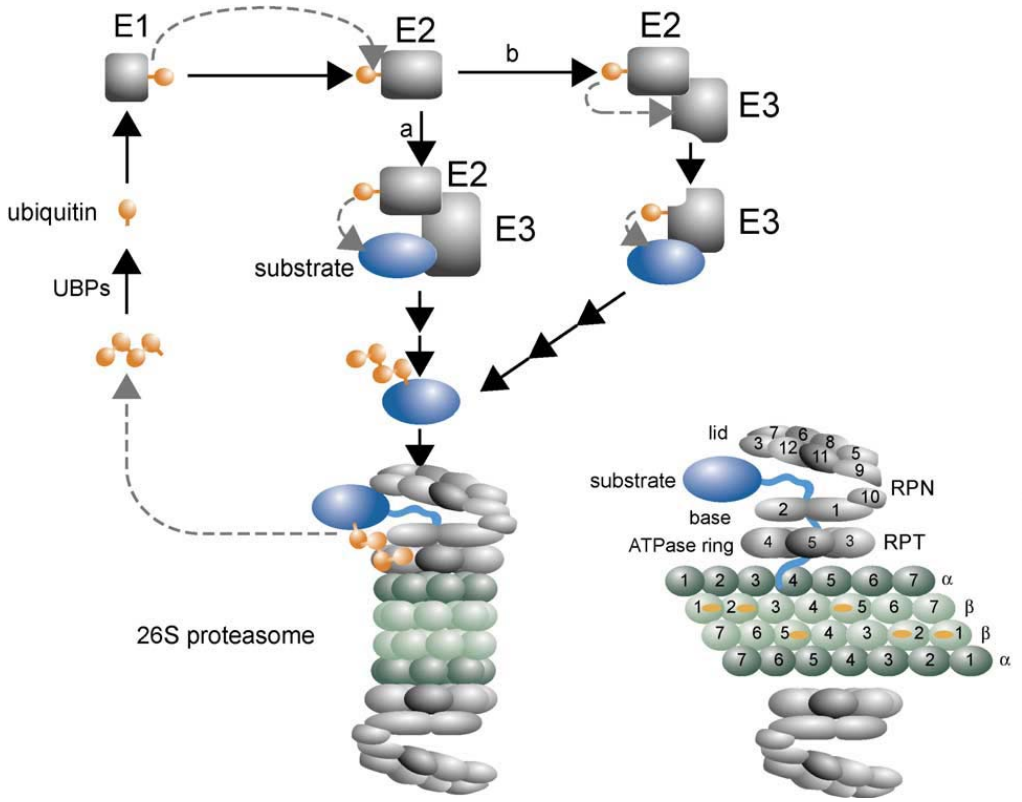

Figure 2. **Ubiquitin proteasome system in yeast.** (Figure reproduced from Wolf and Hilt, 2004)

1.2 Molecular chaperone in the cytoplasm quality control (CQD)

Several lines of evidence suggest the involvement of molecular chaperones in the cytoplasmic protein quality control (Dobson, 2003; McClellan *et al.*, 2005b; Bukau *et al.*, 2006). It was recently discovered that turnover of membrane proteins like the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel localized at the apical surface of polarized epithelial cells, or the CFTR△508 mutant require cytosolic Hsp70 chaperones (Murata *et al.*, 2001; Cyr *et al.*, 2002; Esser *et al.*, 2004). The degradation of CFTR proteins could be discussed in the context of cytosolic quality control because the polytopic CFTR exposes large domains into the cytosol and the misfolding of these domains affects the transport of mature CFTR to the plasma membrane, resulting in cystic fibrosis. As a consequence, all immature ΔF508CFTR molecules and about 60-80% of wild-type CFTR are rapidly degraded by the ubiquitin-proteasome system (Murata *et al.*, 2001; Cyr *et al.*, 2002; Esser *et al.*, 2004). The failure to eliminate misfolded proteins can lead to the formation of potentially toxic aggregates. A number of human diseases are linked to aberrant protein conformations often accompanied by binding of Hsp70 and other chaperones (Kopito, 2000; Dobson, 2003; Goldberg, 2003; McClellan *et al.*, 2005a; Muchowski and Wacker, 2005; Bukau *et al.*, 2006).

1.2.1 Mode of action

The 70-kDa heat shock proteins, or Hsp70s, are central components of the cellular network of molecular chaperones and folding catalysts. Eukaryotic cells contain multiple Hsp70s, which are localized in a variety of cellular compartments including the

cytosol (Hsc70 and inducible Hsp70 of higher organism), mitochondria (Hsp75) and the endoplasmic reticulum (BiP) (Hartl *et al.*, 2002). Classical functions of Hsp70s are prevention of protein aggregation and assistance in protein folding. These functions are based on the transient association of Hsp70 with substrates. Hsp70s recognize short segments of the polypeptide chain, composed of clusters of hydrophobic amino acids (Bukau and Horwich, 1998). Hsp70s are mostly conserved in the first ~530 amino acid residues, with substantially less conservation in the range of residues 530–600, followed by highly variable sequences in the carboxy-terminal 30–50 amino acids (Zhu *et al.*, 1996; Bukau *et al.*, 1998). The N-terminal region of about 44 kDa (380–390 residues) is an ATPase domain which is followed by a central peptide-binding domain. Although the function of the C-terminal variable region has not yet been fully revealed, the region is known to be a binding site for co-chaperones. The extreme carboxy-terminal EEVD motif found in mammalian cytosolic Hsp70s (both the constitutive Hsc70 and the inducible Hsp70) affects the ATPase activity, substrate binding, and interactions with co-chaperones (Zhu *et al.*, 1996; Mayer *et al.*, 2000; Mayer *et al.*, 2005; Morishima, 2005). Recognition of hydrophobic segments is mediated by the central substrate-binding domains of Hsp70 and the substrate binding and release cycle is driven by the switching of Hsp70 between the low-affinity ATP bound state and the high-affinity ADP bound state. In the ATP-bound state, it binds and rapidly releases substrates. Hydrolysis of ATP to ADP catalyzed by intra-molecular ATPase activity leads to the stabilization of the chaperone-substrate complex (Fig. 3).

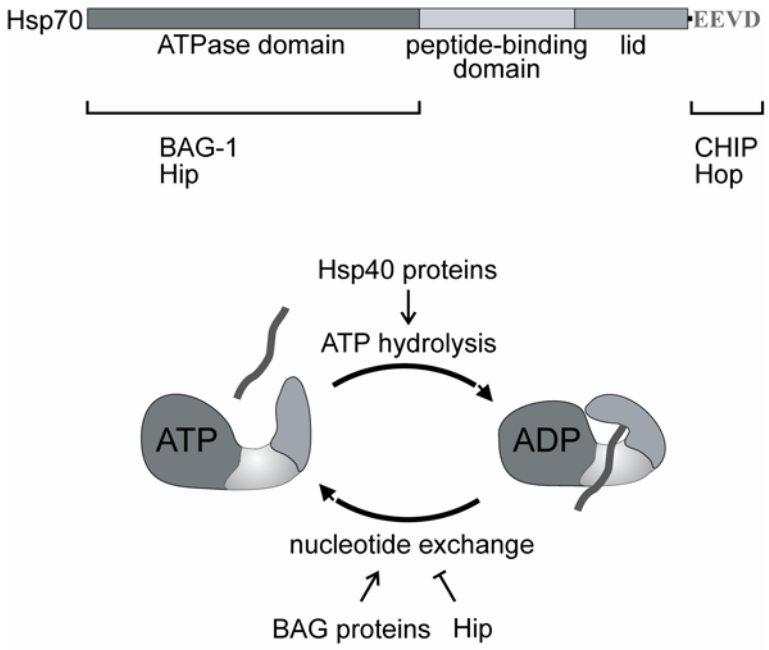

Figure 3. **Domain structure and reaction cycle of Hsp70.** (Figure reproduced from Esser et al, 2004)

Cycles of ATP binding and hydrolysis thus provide the basis for a dynamic interaction of the Hsp70 proteins with non-native polypeptides. However, Hsp70 hydrolyses ATP very inefficiently by itself. Regulatory proteins, so called chaperone cofactors or co-chaperones, are required to induce a physiologically relevant cycling of the chaperone protein (Cheetham and Caplan, 1998; Fan *et al.*, 2003). Cofactors of the Hsp40 family (also termed J proteins due to their founding member, bacterial DnaJ) stimulate the ATP hydrolysis step within the Hsp70 reaction cycle. They play an important role in efficient substrate binding to Hsp70 because they promote the conversion of the chaperone to the ADP-bound state with high substrate affinity. In fact, some Hsp40 proteins, such as bacterial DnaJ and yeast Ydj1, can prevent aggregation by themselves, through ATP-independent transient and rapid association with substrates (Cheetham *et al.*, 1998; Fan

et al., 2003). Their own chaperone activity may allow these Hsp40 proteins to target Hsp70 to exposed hydrophobic stretches of a substrate protein and simultaneously initiate a functional chaperone cycle.

ATP dependent cycling of Hsp70 requires a second set of regulatory co-chaperones, Hip and Bag1 to act as nucleotide exchange factor. Hip (Hsc70 interacting protein) binds to the ATPase domain and increases the chaperone activity of Hsp70 by stabilizing the ADP-bound state (Hohfeld *et al.*, 1995; Frydman and Hohfeld, 1997; Hohfeld and Jentsch, 1997; Hohfeld *et al.*, 2001; Alberti *et al.*, 2003; Alberti *et al.*, 2004). In contrast, Bag1 inhibits the chaperone activity of Hsp70 in a manner competitive with Hip by facilitating premature release of the unfolded substrate by accelerating nucleotide exchange. At the same time, the cofactor Hop (Hsp70/Hsp90-organizing protein) associates with Hsp70. The 60-kDa protein Hop (yeast Sti1p) has been identified as a protein involved in the regulation of the heat shock response to stimulate the ATPase activity of yeast Hsp70 (Nollen *et al.*, 2001; Kabani *et al.*, 2002; Wegele *et al.*, 2003; McClellan *et al.*, 2005a). Hop interacts via its three TPR domains with the C-terminal EEVD motif of Hsp70 and Hsp90 proteins. Another important TPR domain-containing protein is the 35-kDa protein CHIP (carboxyl terminus of Hsc70-interacting protein) in mammal (Connell *et al.*, 2001; Demand *et al.*, 2001; Cyr *et al.*, 2002; Alberti *et al.*, 2004). The CHIP protein was initially identified in a screen for human proteins that possess a tetratricopeptide repeat (TPR) domain. In addition to the amino terminal TPR domain, CHIP possesses a U-box at its carboxyl terminus. The U-box is structurally related to RING-finger domains found in many ubiquitin ligases, which suggested a function of CHIP in ubiquitin conjugation. Indeed, CHIP supplies its U-box for binding to E2 ubiquitin-conjugating enzymes of the Ubc4/5 family and acts

as an E3 ubiquitin ligase during the ubiquitination process of substrates (Demand *et al.*, 2001; Jiang *et al.*, 2001; Murata *et al.*, 2001). CHIP apparently shifts the mode of action of chaperones from protein folding to protein degradation. Clearly, it is not involved in the productive folding of chaperone substrates. In addition to the conserved Bag domain, the BAG family members possess a ubiquitin like domain that seems to cooperate functionally with CHIP to mediate targeting to the proteasome (Demand *et al.*, 2001; Sondermann *et al.*, 2001; Sondermann *et al.*, 2002; Alberti *et al.*, 2003). Bag-1 uses the integrated ubiquitin like domain for an association with the proteasome. Therefore Bag-1 can act as a coupling factor between Hsp70 and the proteasome. Bag-1 interacts with the amino terminal ATPase domain of Hsp70, whereas CHIP binds to the carboxyl terminal EEVD motif in the chaperone. A functional chaperone system is formed only when Hsp70 tightly cooperates with regulatory cofactors that modulate the ATPase cycle of the chaperone or mediate targeting to other proteins and protein complexes. In particular, the large diversity of co-chaperones present in the eukaryotic cytosol seems to enable Hsp70 to fulfill its multiple functions in this compartment (Esser *et al.*, 2004).

1.3 Aim of this work

Protein quality control by molecular chaperones includes recognition of misfolding, prevention of protein aggregation and facilitation of refolding of partially unfolded proteins (Goldberg, 2003; Kleizen *et al.*, 2004). Misfolded secretory proteins are recognized in the endoplasmic reticulum (ER), prevented from continuing along the secretory pathway, retrotranslocated to the cytoplasmic side of the ER, polyubiquitinated and delivered to the proteasome for degradation. Interestingly, cytoplasmic chaperones of Ssa family are necessary for the removal of certain membrane proteins. However, they are dispensable for the elimination of the mutant Sec61-2 protein as well as for the degradation of soluble proteins like mutant α-factor. In this study, a set of structurally different misfolded proteins sharing the same ER-degradation signal were used to demonstrate mechanistic diversity and to reveal differences in chaperone requirements during ERAD. The degradation requirements of the cytoplasmically located CPY* derivative ΔssCPY*-GFP were investigated during the study of the delivery mechanism of misfolded ER substrates to the proteasome. The elimination of ΔssCPY*-GFP does not require the Cdc48p-Ufd1p-Npl4p AAA-ATPase complex or the UBA-UBL proteins Dsk2p and Rad23p (Medicherla *et al.*, 2004). This pointed to a completely different recognition and delivery mechanism for this misfolded ER import defective protein.

Recently, it has been found in mammalian cells that the efficiency of protein compartmentalization into the secretory pathway is far from perfect. Due to inefficient signal sequence recognition, inefficient translocation into the ER and leaky ribosomal scanning, the efficiency of segregation to the ER was shown to vary considerably

(Levine *et al.*, 2005). This raises the question of the fate of these remnant proteins mislocalized to the cytoplasm.

2. Materials and Methods

2.1 Materials

2.1.1 Media for yeast cultures

Standard yeast rich (YPD) and minimal (CM) media were prepared as described (Guthrie and Fink, 1991; Sambrook, 2001). Geneticin (G418) resistant cells were grown on YPD plates containing 200 µg/ml of G418. Ura$^-$ cells were selected on solid synthetic complete medium containing 5-FOA at 1 mg/ml.

2.1.2 Media for *E.coli* cultures

Ampicillin (Stock solution of 50 mg/ml) was added to the medium to a final concentration of 50 µg/ml.

2.1.3 Solutions

Solution	**Components**
Agarose Gel	1-2 % (w/v) Agarose in TAE-Buffer, pH 7.5 0.5 µg/ml ethidiumbromide
Alkaline lysis buffer	925µl 2M NaOH 75µl β- Mercaptoethanol
Blocking-Buffer	5% (w/v) non-fat dry milk in PBST
Breaking Buffer	50 mM Tris-HCl pH7.5, 6 M Urea, 1% SDS, 1 mM EDTA
Chase Medium	Same as labeling medium w/ 0.6 % of L-methionine and 0.2 % of BSA

IP Buffer	50 mM Tris-HCl pH 7.5, 190 mM NaCl, 1.25 % TritonX-100 (v/v), 6 mM EDTA
IP Buffer w/o Triton-X 100	50 mM Tris-HCl pH 7.5, 190 mM NaCl, 6 mM EDTA
Labeling Medium	0.17 % Yeast Nitrogen Base w/o ammonium sulfate and w/o amino acids, 0.1 % D-glucose; 0.002 L-adenine, uracil, L-tryptophan, L-histidine, 0.003 % L-arginine, L-tyrosine, L-lysine, L-leucine, 0.005 % L-phenylalanine, 0.01 % L-glutamic acid, L-aspartic acid, 0.015 % L-valine, 0.02 % L-threonine, 0.04 % L-serine
Solubilization buffer	1 % SDS, 50 mM Tris-Cl pH 7.5
Oxalyticase stock solution	5 mg/ml oxalyticase 50 mM Na-Phosphate buffer , pH 7.4 50% Glycerol
Oxalyticase-buffer	0.7 M Sorbitol 50 mM Tris-HCl pH 7.5
PBST	16 mM Na_2HPO_4 4 mM NaH_2PO_4 100 mM NaCl 0.5 % (v/v)Tween 20
PMSF	0.8 M PMSF in DMSO
PS 200	200 mM Sorbitol, 20 mM PIPES 5 mM $MgCl_2$, pH 6.8
Resolving Gel Buffer	1.5 M Tris-HCl pH 8.8
Sorbitol lysis buffer	0.7 M sorbitol 50 mM Tris-HCl pH 7.5

	2 mM PMSF (Freshly Added) 1 μg/ml pepstatin-A (Freshly Added)
Stacking Gel Buffer	0.5 M Tris-HCl pH 6.8
Stripping Buffer	62.5 mM Tris-HCl pH7.5 2% (w/v) SDS 100 mM β-mercaptoethanol
TE buffer	10 mM Tris, 1.0 mM EDTA, pH 7.5
Transfer-Buffer	12 mM Tris 96 mM Glycine 20% (v/v) Methanol
Tris/Sulfate DTT	0.1M Tris-H_2SO_4 pH 9.4 20 mM DTT (freshly added)
Urea buffer for SDS–PAGE	200 mM Tris-HCl pH 6.8 8 M Urea 5% (w/v) SDS 0.1 mM EDTA 0.03% Bromophenol blue 1 mM β- mercaptoethanol
Running buffer pH 8.3	25 mM Tris 192 mM glycine 0.1% (w/v) SDS
TAE- Buffer, pH7.5	40 mM Tris-Acetate 2 mM EDTA
Washing buffer for ubiquitination	20 mM NaN_3 2 mM PMSF (Freshly Added) 20 mM NEM (Freshly Added)

2.1.4 Chemicals

Chemical	Supplier
β-mercaptoethanol	Merck, Darmstadt
Acetic acid	Riedel-De Haën, Seelze
Acetone	Riedel-De Haën, Seelze
Acrylamide and bisacrylamide solutions	Genaxxon BioScience Stafflangen, Schröder, Stuttgart
Agarose NEEO	Roth, Karlsruhe
Ammonium persulfate (APS)	Genaxxon Bioscience, Stafflangen
Ampicillin	Genaxxon Bioscience, Stafflangen
Bacto™ agar	BD, Sparks, USA
Bacto™ peptone	BD, Sparks, USA
Bacto™ tryptone	BD, Sparks, USA
Bromophenol blue	Riedel-De Haën, Seelze
BSA	New England Biolabs, USA
Calcium chloride	Sigma-Aldrich Chemie, Steinheim
Chloroform	Fisher Scientific, Leicestershire, UK
Cycloheximide	Sigma-Aldrich Chemie, Steinheim
D-glucose	Roth, Karlsruhe
Dithiothreitol (DTT)	Roth, Karlsruhe
DMSO	Merck, Darmstadt
DNA standard (1 kb DNA ladder)	Roche, Mannheim
Ethanol	Roth, Karlsruhe
Ethidiumbromide	Sigma-Aldrich Chemie, Steinheim
EDTA	Sigma-Aldrich Chemie, Steinheim

FITC (fluorescein isothiocyanate)	Sigma-Aldrich Chemie, Steinheim
Glass beads (0.5-mm)	B. Braun Biotech, Melsungen
Glycerol	Riedel-De Haën, Seelze
Herring sperm DNA	Promega, Madison, USA
Isopropanol	Merck, Darmstadt
L-alanine	Sigma-Aldrich Chemie, Steinheim
L -arginine	Sigma-Aldrich Chemie, Steinheim
L -asparagine	Sigma-Aldrich Chemie, Steinheim
L -aspartic acid	Sigma-Aldrich Chemie, Steinheim
L -cysteine	Sigma-Aldrich Chemie, Steinheim
L -glutamic acid	Sigma-Aldrich Chemie, Steinheim
L -glutamine	Sigma-Aldrich Chemie, Steinheim
L-glycine	Roth, Karlsruhe
L -histidine	Sigma-Aldrich Chemie, Steinheim
L -isoleucine	Sigma-Aldrich Chemie, Steinheim
Lithium acetate	Sigma-Aldrich Chemie, Steinheim
L -leucine	Sigma-Aldrich Chemie, Steinheim
L -lysine	Sigma-Aldrich Chemie, Steinheim
L -methionine	Sigma-Aldrich Chemie, Steinheim
L -phenylalanine	Sigma-Aldrich Chemie, Steinheim
L -proline	Sigma-Aldrich Chemie, Steinheim
L -serine	Sigma-Aldrich Chemie, Steinheim
L -sorbitol	Roth, Karlsruhe
L -threonine	Sigma-Aldrich Chemie, Steinheim

L -tryptophane	Sigma-Aldrich Chemie, Steinheim
L-tyrosine	Sigma-Aldrich Chemie, Steinheim
L-valine	Sigma-Aldrich Chemie, Steinheim
Magnesium chloride	Roth, Karlsruhe
Magnesium sulfate	Roth, Karlsruhe
Methanol	Sigma-Aldrich Chemie, Steinheim
p-amino benzoic acid	Sigma-Aldrich Chemie, Steinheim
Phenol, saturated with TE (Roti®-Phenol)	Roth, Karlsruhe
Phenyl-methyl-sulfonyl fluoride (PMSF)	Merck, Darmstadt
Polyethylene glycol (PEG) 3350	Sigma-Aldrich Chemie, Steinheim
Ponceau S	Sigma-Aldrich Chemie, Steinheim
Potassium acetate	Merck, Darmstadt
Potassium chloride	Merck, Darmstadt
Potassium dihydrogen phosphate	Merck, Darmstadt
Potassium hydrogen phosphate	Roth, Karlsruhe
Protease inhibitor cocktail tablets	Roche Diagnostics, Mannheim, Germany
Protein A Sepharose^{TM} CL-4B	Amersham Biosciences, Uppsala, Sweden
SeeBlue^{TM} pre-stained standard	Novex, San Diego, USA
Sodium acetate	Merck, Darmstadt
Sodium azide	Riedel-De Haën, Seelze
Sodium chloride	Roth, Karlsruhe
Sodium dihydrogen phosphate	Roth, Karlsruhe
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Chemie, Steinheim
Sodium hydrogen phosphate	Roth, Karlsruhe

Sodium hydroxide	Merck, Darmstadt
TEMED	Merck, Darmstadt
Trichloro acetic acid (TCA)	Roth, Karlsruhe
Tris	ICN Biomedicals, Aurora, USA
TritonX-100	Roth, Karlsruhe
Tween-20	Sigma-Aldrich Chemie, Steinheim
Urea	Roth, Karlsruhe
Yeast extract	Difco, Michigan, USA
Yeast nitrogen base w/o amino acids	Difco, Michigan, USA
Yeast nitrogen base w/o amino acids and ammonium sulfate	Difco, Michigan, USA

2.1.5 Miscellaneous materials

Material	**Supplier**
Filter paper GB002	Schleicher und Schuell, Dassel
Hyperfilm^{TM} ECL	Amersham Biosciences, Uppsala, Sweden
Nitrocellulose membranes pH 7.5	Schleicher und Schuell, Dassel, PALL Life Sciences, Pensacola, USA
Membrane Filter	Millipore, Billerica, Mass., USA
Electroporation cuvettes	EquiBio, Kent, UK
Autoradiography Film Biomax MR	Kodak, Stuttgart, Germany

2.1.6 Laboratory equipment

Equipment	**Suppplier**
Agarose gel electrophoresis apparatus	Bio-Rad, Hercules, USA

Biofuges fresco and pico	Heraeus, Hanau
Centrifuge 5417 C and 5804R	Eppendorf, Hamburg
Centrikon H-401	Kontron Instruments
Developer machine OPTIMAX Typ TR	MS Laborgeräte, Heidelberg
Heating block	Liebisch, Bielefeld
Incubator	Heraeus, Hanau
Ion exchanger Milli-Q Plus	Millipore, Eschborn
Lab-shaker	Adolf Kuhner AG, Switzerland
Multi vortexer IKA-VIBRAX VXR	Janke & Kunkel
Optima™ TLX Ultracentrifuge	Beckman, Palo Alto, California
Overhead rotator REAX2	Heidolph Instruments, Schwabach
pH Meter CG 832	Schott, Hofheim
Pipettes (2-1000µl)	Gilson
Power supply units Model 200	Bio-Rad, Hercules, USA
Robocycler® Gradient 40	Stratagene, La Jolla, USA
SDS-PAGE apparatus	Bio-Rad, Hercules, USA
Semidry blot chamber	ITF Labortechnik, Wasserburg
Shakers, various sizes	A. Kühner, Birsfelden, Switzerland
Spektrophotometer Jasco V-530	Jasco, Germany
Spektrophotometer Novaspec II	Pharmacia Biotech, Uppsala, SWE
Stirrer lkamag® REO	IKA®-Labortechnik, Staufen i. Br.
Thermomixer 5437	Eppendorf, Hamburg
Vortexer Vibrofix VF 1 and VF2	IKA®-Labortechnik, Staufen i. Br.
Water bath W13/F3	Haake, Karlsruhe

2.1.7 Enzymes

Enzyme	Supplier
SuRe/Cut™ System endonucleases,	Roche, Mannheim
T4-DNA-Ligase	Invitrogen, Carlsbad, USA
Pwo-DNA-polymerase	PEQLAB Biotechnologie, Erlangen
PfuUltra™ high-fidelity DNA-polymerase	Stratagene, La Jolla, USA
Oxalyticase	Enzogenetics, Corvallis, USA
Proteinase K	Sigma-Aldrich Chemie, Steinheim

2.1.8 Antibodies

Anti body	Dilution	Reference
Mouse anti CPY	1:10000 for IB	Molecular Probes
Mouse anti HA	1:500 for IP 1:10000 for IB	Babco
Mouse anti PGK	1:10000 for IB	Molecular Probes
Mouse anti Ubiquitin	1: 2000 for IB	Babco
Rabbit anti CPY	1:200 for IP	(Finger *et al*., 1993)
Rabbit anti Kar2	1:10000 for IB	R. Schekman
Rabbit anti Sec61	1:10000 for IB	T. Sommer
Anti rabbit IgG, HRPO conjugated	1:10000 for IB	Sigma-Aldrich Chemie
Goat anti mouse IgG, HRPO conjugated	1:10000 for IB	Jackson Immuno Research Laboratories

2.1.9 Reagent kits

Reagent kit	Supplier
ECL™ Kit	Amersham, Little Chalfont, UK
QIAEXⅡ Gel Extraction Kit	Qiagen, Hilden
QIAprep Spin Miniprep Kit	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
QuickChange® II XL Site-Directed Mutagenesis Kit	Stratagene, La Jolla, USA

2.1.10 Organism

2.1.10.1 *Saccharomyces cerevisiae* strains

Strain	Genotype	Source
YWO1	*Mat α ura3-52 leu2-3,2-112 his3 Δ200 lys2-801 trp1-1*	(Seufert *et al*, 1990)
YWO23	*Mat α ura3-52 leu2-3,2-112 his3 Δ200 lys2-801 trp1-1 Δubc4::HIS3 Δubc5::LEU2*	(Seufert *et al*, 1990)
YPH499Y	*Mat a ura3-52 leu2-1 his3Δ200 trp1-63 lys2–801 ade2-101 prc1–1*	(Hiller *et al.*, 1996)
CMY762Y	*Mat a cim3–1 ura3–52 leu2-1 his3 Δ200 prc1–1*	(Hiller *et al.*, 1996)
W303-1C	*Mat α ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 prc1-1*	(Knop *et al.*, 1996a)
YRP160	W303-1C *Mat a kar2-159*	(Plemper *et al.*, 1997)
YPK001	YRP160 *Δprc1::KAN^R*	This study
YPK002	W303-1C *Δsnl1::KAN^R*	This study

YPD5	W303–1C *Δydj1–2::HIS3 LEU2::ydj1–151*	(Taxis *et al.*, 2003)
YPD21	*Mat α his3–11, 15 leu2–3, 112 ura3–52 trp1–81 lys2 prc1–1 Δssa2::LEU2 Δssa3::TRP1 Δssa4::LYS2*	(Taxis *et al.*, 2003)
YPD22	YPD21 *ssa1–45 Δssa2::LEU2 Δssa3::TRP1 Δssa4::LYS2*	(Taxis *et al.*, 2003)
YPK003	YPD21 $SSA1^{K521R}$	This study
YPK004	YPD21 $SSA1^{K536R}$	This study
YPK005	YPD21 $SSA1^{K521R/K536}$	This study
YCT397	*Mat a leu2-3,112 ura3-52 ade1-100 his4-519 prc1-1*	(Jarosch *et al.*, 2002)
YCT415	YCT397 *ufd1-1*	(Jarosch *et al.*, 2002)
YCT595	W303-1C $\Delta hlj1::KAN^R$	(Taxis *et al.*, 2003)
YCT598	W303-1C $\Delta jid1::KAN^R$	(Taxis *et al.*, 2003)
YRH25	W303-1C $\Delta cwc23::KAN^R$	(Taxis *et al.*, 2003)
MHY501	*Mat α his3-200 leu2-3,112 ura3-52 lys2-801 trp1-1*	(Swanson *et al.*, 2001)
MHY1631	MHY501 *Δssm4/doa10::HIS3*	(Swanson *et al.*, 2001)
MHY1669	MHY501 *Δhrd1/der3::LEU2*	(Swanson *et al.*, 2001)
MHY1703	MHY501*Δhrd1/der3::LEU2 Δssm4/doa10::HIS3*	(Swanson *et al.*, 2001)
YRH023	W303-1C $\Delta hsp104::KAN^R$	(Taxis *et al.*, 2003)
YRH030	W303-1C *Δsti1-1::HIS3*	(Taxis *et al.*, 2003)
YRH050	W303-1C $\Delta hsc82::KAN^R$ $hsp82^{G170D}$	(Taxis *et al.*, 2003)

Y406-C	*Mat α ura3-52 leu2-3,112 his3-11,15 lys2 trp1-1 prc1-1*	(Deak, 1998)
Y420-C	Y406-C *Δssb1::LEU2 Δssb2::HIS3*	(Deak, 1998)
W303-1B	*Mat α ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*	(Chiang and Schekman, 1991)
AGC14	*Mat α ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1can1-100Δhsp26::LEU2 Δhsp42::HygB^R*	(Cashikar *et al.*, 2005)
BY4743	*Mat α/a his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2,MET15/met15Δ0, ura3Δ0/ura3Δ0*	EUROSCARF
BY4743 *Δprc1*	BY4743 *Δprc1::kanMX4/Δprc1::kanMX4*	EUROSCARF
BY4743 *Δsnl1*	BY4743 *Δsnl1::kanMX4/Δsnl1::kanMX4*	EUROSCARF
BY4743 *Δsse1*	BY4743 *Δsse1::kanMX4/Δsse1::kanMX4*	EUROSCARF

2.1.10.2 *E.coli* strains

***E. coli* strain**	**Genotype**	**Remarks**
DH5α	*F- Φ 80d lac Z ΔM15 (argF-lacZYA) U169 end A1recA1 hsd R17(rk-, mk+) deo R thi- 1 supE44 λ- gyrA96 relA1 Δ*	Gibco-BRL, Invitrogen

XL10-Gold®	*Tetr Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 thi-1 recA1 gyrA96 relA1 lac Hte [F′ proAB lacIqZ ΔM15 Tn10(Tetr) Amy Camr]*	Stratagene, La Jolla, USA

2.1.11 Synthetic Oligonucleotide primers

Oligo name	**Sequence**
5'Sph1	TCAACTTAAAGTATACATACGCTGCATGCATGATCTCATTG CAAAGACC
3' Sph1	CGGTCTTTGCAATGAGATCATGCATGCAGCGTATGTATACT TTAAGTT
5'GFP-Sph1	AAGCTTGCATGCATGGCTAGCAAAGGAGAAGAACTC
3'GFP-Sph1	AAGCTTGCATGCGCAGCCGGATCCTTTGTATAGTTC
5'seqCPY	GCACGAGATAAGAATGCC
Z3'CPY-Ecor1	CGGAATTCATTGTACTTACAAACTCG
5'Bsu361(F10)	CCGGGTCCTCAGGTGTTTCC
5'CPY-BstAP1(F11)	GGTGGCAATTTGTGCTACCCAA
3'CPY-Ecor1(R10)	CCGGAATTCTCAGCACTGAGCAGCGTAAT
5'GFP_{uv}-Mlu1	CTACAAGACGCGTGCTGAAGTCAAG
3'GFP_{uv} -Ecor1	CTGCAGGAATTCCTACACATTGATC
F521m	AGATGGTTGCTGAAGCCGAACGTTTCAAGGAAGAAGATGA AAAGGA
R521m	TCCTTTTCATCTTCTTCCTTGAAACGTTCGGCTTCAGCAACC ATCT

P1a	GAATCTCAAAGAATTGCTTCTAGAAACCAATTGGAATC
P1a-R	GATTCCAATTGGTTTCTAGAAGCAATTCTTTGAGATTC
SNL1 5’ Primer A	GACGAATATAAGGTCAAAAGCTTCA
SNL1 3’ Primer B	CTTGTTCTTTTTAGAAGCCCTCTTT
SNL1 5’ Primer C	TGAAGTTGCTGATTGAGTTAGACAG
SNL1 3’ Primer D	TTTATTTTGGTATGATTTTAGGCGA
5’ Kan C	TGATTTTGATGACGAGCGTAAT
3’ Kan B	CTGCAGCGAGGAGCCGTAAT

2.1.12 Plasmids

The protein of interest is expressed under the control of its own promoter unless specifically mentioned.

Name	Description	Reference
pRS316	Centromeric yeast/*E.coli* shuttle vector with URA3 marker	(Christianson et al., 1992)
pMA1 (TDH3 promoter)	pRS316 constitutively expressing CPY* and transmembrane domain followed by GFP	(Taxis et al., 2003)
pCT67 (TDH3 promoter)	pRS316 constitutively expressing CPY* with a transmembrane domain	(Taxis et al., 2003)

pRS316-ΔssCPY*-GFP	pRS316 expressing cytosolic (Δss)CPY*- GFP	(Medicherla et al., 2004)
pRS316-ΔssCPY*	pRS316 expressing cytosolic (Δss)CPY*	This study (Cloned by Z. Kostova)
pRS316-ΔssCPY (pSH10)	pRS316 expressing cytosolic (Δss)CPY	This study
pRS316-ΔssCPY*-HA_3 (pSH11)	pRS316 expressing cytosolic (Δss)CPY*-HA_3	This study
pRS316-ΔssCPY-HA_3 (pSH12)	pRS316 expressing cytosolic (Δss)CPY-HA_3	This study
pRS316-ΔssGFP-CPY* (pSH8)	pRS316 expressing cytosolic (Δss)GFP-CPY*	This study
pRS316-ΔssCPY*-GFP_{uv} (pNB001)	pRS316 expressing cytosolic (Δss)CPY*-GFP_{uv}	(Bolender, 2005)
pRS426-ΔssCPY*-GFP (pSH13)	pRS426 over-expressing cytosolic (Δss)CPY*- GFP	This study
pRS316-ΔssCPY*-GFP_{uv}-cODC (pSH14)	pRS316 expressing a fusion protein consisting of ΔssCPY* and GFPuv-cODC	This study
pRS316-ΔssCPY*-GFP_{uv}-cODC-C441A (pSH15)	pRS316 expressing a fusion protein consisting of ΔssCPY* and GFPuv-cODC-C441A	This study
pRS313- ΔssCPY*-GFP	pRS313 expressing cytosolic	This study

(pSH16)	(Δss)CPY*- GFP	
YIpSSA1BH	YIp5 plasmid containing the 7kb *HindIII-BamH1* fragment of SSA1	E. Craig, Department of Biomolecular Chemistry, University of Wisconsin
pRS306-SSA1 (pSH4)	Integrative pRS306 plasmid containing 2.8kb *Xba1-Spe1* fragment of *SSA1*	This study
pRS306-SSA1^{K521R} (pSH5)	Integrative pRS306 plasmid containing a mutation of Lys521 to Ala521 in *SSA1*	This study
pRS306-SSA1^{K536R} (pSH6)	Integrative pRS306 plasmid containing a mutation of Lys536 to Ala536 in *SSA1*	This study
pRS306-SSA1$^{K521R/K536R}$ (pSH9)	Integrative pRS306 plasmid containing double mutation of Lys521 to Ala521 and Lys536 to Ala536 in *SSA1*	This study

2.2 Methods

Yeast genetics experiments were carried out using standard methods. (Guthrie *et al.*, 1991; Sambrook, 2001).

2.2.1 Molecular biological methods

Standard conditions were used to generate PCR fragment and recombinant DNA-techniques and transformation of plasmids into *E.coli* were done as described (Sambrook, 2001).

2.2.1.1 Site directed mutagenesis

In vitro site directed mutagenesis was performed with the QuickChange® II XL Site-Directed Mutagenesis Kit according to manufacture's instructions. Briefly, the mutation was introduced in a specific site by the use of two synthetic oligonucleotide primers, both containing the desired mutation. The two primers are complementary to each other and to opposite strands of the plasmid DNA and are extended during temperature cycling by *PfuUltra* High Fidelity DNA polymerase, without primer displacement. The extension cycle generates a mutated plasmid containing staggered nicks. After the thermal cycling the product is treated with *DpnI*. This enzyme is specific for methylated and hemimethylated DNA and is used for digestion of the parental DNA template. The plasmid DNA containing the desired mutations was then transformed into XL10-Gold® ultracompetent cells or in DH5α cells. Both strains are able to repair the nick and to replicate the plasmid. Cells were plated on LB containing the selective antibiotic.

2.2.1.2 Construction of plasmids

A 1.2kb partial fragment of wild type *PRC1* was amplified from pYEP13/*PRC1* using the primer pair F10 and z3'CPY-Ecor1 and inserted into pRS316-ΔssCPY* between the *Bsu361* and *EcoR1* restriction sites, generating pRS316-ΔssCPY. The DNA of cytoplasmically localized, N-terminally GFP fused CPY* (ΔssGC*) was cloned in two steps. First, the *Sph1* restriction site was introduced to the end of the CPY promoter in pRS316-ΔssCPY* by QuickChange® II XL Site-Directed Mutagenesis Kit using the primer pair 5'Sph1 and 3'Sph1 to generate pZK116m. Then the 0.7kb GFP DNA fragment was amplified from plasmid pRS316-ΔssCPY*-GFP by the primer pair 5'GFP-Sph1 and 3'GFP-Sph1 was cloned into the *Sph1* restriction site of pZK116m, generating plasmid1 pRS316-ΔssGFP-CPY*. The 3.4kb DNA fragment encoding ΔssCPY*-GFP was sub-cloned from plasmid pRS316-ΔssCPY*-GFP into the 2μ plasmid pRS426 and plasmid pRS313 between the *Cla1* and *EcoR1* restriction sites, respectively. The 0.5kb DNA fragment of GFP_{uv}-cODC or GFP_{uv}-cODC-C441A from $p416P_{ADH}$-GFP_{uv}425cODC or $p416P_{ADH}$-GFP_{uv}425cODC-C441A(Hoyt et al., 2003), respectively, was PCR-amplified by the primer pair 5'GFP_{uv}-Mlu1 and 3'GFP_{uv}-Ecor1 and cloned between the *Mlu1* and *EcoR1* restriction sites of pRS316-ΔssCPY*-GFP_{uv}, yielding pRS316-ΔssCPY*-GFP_{uv}-cODC or pRS316-ΔssCPY*-GFP_{uv}-cODC-C441A, respectively. The C-terminal HA_3 tag to CPY* was PCR amplified from plasmid pCT42 which encodes CPY*-HA_3 using the primer pair F10 and R10 for ΔssCPY*-HA_3 or F11 and R10 for ΔssCPY-HA_3. The PCR products were cloned into between the *Bsu361* and *Ecor1* sites of pRS316-ΔssCPY* or between the *BstAP1* and *Ecor1* sites of pRS316-ΔssCPY, yielding pRS316 expressing ΔssCPY*-HA_3 and ΔssCPY-HA_3, respectively. A 2.8Kb *Xba1-Spe1* excision fragment including the *SSA1* allele from

plasmid YIpSSA1BH (E.Craig) was sub-cloned into pRS306 between *Xba1* and *Spe1*, creating integrative pRS306-SSA1. Two mutations of *SSA1* alleles, $SSA1^{K521R}$, $SSA1^{K536R}$, were created using the QuikChange® II XL Site-Directed Mutagenesis Kit with pRS306-SSA1 as the template. The primer pairs used were as followed: F521m and R521m for $SSA1^{K521R}$; P1a and P1a-R for $SSA1^{K536R}$. The mutagenesis resulting in plasmid pRS306-$SSA1^{K521R}$ had an *Acl1* restriction site in K521R and pRS306-$SSA1^{K536R}$ had a *Xba1* restriction site in K536R. Additionally, plasmid pRS306-$SSA1^{K521R}$ was used as the template to create the $SSA1^{K521R/K536R}$ mutation using the primer pair P1a and P1a-R, creating pRS306- $SSA1^{K521R/K536R}$.

2.2.1.3 Construction of yeast strains

The *prc1-1* allele in YRP160 was disrupted by PCR amplification of the Δ*prc1*::KAN^R fragment from BY4743 Δ*prc1*::KAN^R (EUROSCARF, Frankfurt) using the primer pair 5'seqCPY and z3'CPYEcoRI and transformation of the obtained DNA fragment into YRP160, yielding YPK001 (Δ*prc1 kar2-159*). The *SNL1* gene in W303-1C was disrupted by PCR amplification of the Δ*snl1*::KAN^R fragment from strain *BY4743* Δ*snl1*::KAN^R (EUROSCARF, Frankfurt) using the primer pair SNL1 5' and SNL1 3' and transformation of the obtained DNA fragment into W303-1C, yielding YPK002 *(W303-1C Δsnl1)*. Correct integration of the disrupting DNA was confirmed by PCR analysis and Southern blotting. Two mutations of the *SSA1* gene, $SSA1^{K521R}$, $SSA1^{K536R}$, were integrated into the *SSA1* locus of YPD21 through recombination of the linear fragment of pRS306- $SSA1^{K521R}$ and pRS306-$SSA1^{K536R}$ using *Van911* restriction enzyme, generating YPK003 and YPK004, respectively. The double mutation of $SSA1^{K521R/K536R}$ was introduced into YPD21 through the recombination of the linear

fragment of pRS306- *SSA1*$^{K521R/K536R}$ generated by *Van911* restriction enzyme, consequently yielding YPK005. Correct integration of the mutation alleles was verified by sequencing of PCR products.

2.2.2 Biochemical methods

2.2.2.1 Cycloheximide decay analysis

Cells were grown at 30℃ to logarithmic phase in synthetic complete medium. Temperature sensitive strains were shifted to the restrictive temperature of 37℃ for 60 min. Cycloheximide was added (0.5 mg/ml) and 2 OD_{600} of cells were taken at the indicated time points. Cell extracts were prepared by alkaline lysis (Hiller *et al.*, 1996; Taxis *et al.*, 2003) and subjected to SDS-PAGE followed by immunodetection.

2.2.2.2 Alkaline lysis of yeast whole cell extracts

Cells were harvested at 14000 rpm for 1 min. The cell pellet was resuspended in 1 ml of dH_2O and freshly prepared 150 µl of NaOH and β-mercaptoethanol mix (925 µl of 2 M NaOH and 75 µl of 13.3 M β-mercaptoethanol) was added and kept on ice for 10 min with brief vortexing every 3 min. 150 µl of TCA (55%) was added to the samples and kept on ice for 10 min. Cells were centrifuged at 14000 rpm in a table top centrifuge for 10 min, supernatant was removed. Pellet was washed once with 500 µl of ice-cold acetone. Pellet was resuspended in 100 µl of Urea loading buffer completely by shaking at 65°C.

2.2.2.3 SDS-PAGE and Western Blotting

SDS-PAGE analysis was performed for the separation of proteins and Western blotting and immuno detection were followed as described (Coligan *et al.*, 1995). Briefly, samples were run along with the standard protein maker. When the bromophenol band was electrophoresed out, the protein samples were transferred from the gel to the nitrocellulose membrane in a semidry blot chamber using the transfer buffer. After completing transfer, nitrocellulose membranes were blocked using 10% non-fat dry milk powder in PBST buffer by incubating the membranes over night at 4℃. After membranes were washed two times with PBST buffer for 10 min, they were incubated with required primary antibody (for dilutions of individual antibody see antibody section) for 1hr at RT, followed by washing the membranes two times with PBST buffer for 10min. Thereafter the membranes were incubated for 1 hr with the secondary antibody, which is conjugated to Horse Radish Peroxidase. Immunodetection was carried out using ECL-kit according to manufacture's instruction. When it was required, the membranes were reprobed with a second antibody after being incubated with Stripping buffer at 60℃ for 25 min with occasional agitation.

2.2.2.4 Preparation of yeast spheroplasts

50 OD_{600} of yeast cells were collected and resuspended in TRIS-Sulfate DTT solution and incubated for 10 min at 30°C. Cells were spun down (2000 rpm, 5 min.) and resuspended in 3 ml of oxalyticase buffer with addition of oxalyticase stock solution to a final oxalyticase of 5 µg per 1 OD_{600} of cells. Cells were incubated for 30 min 30°C with light shaking. Spheroplasts were collected by centrifugation (1500 g, 5 min.) and resuspended in 2 ml of ice-cold PS200 buffer containing 2 mM PMSF. Lysis of

spheroplasts was carried out by re-suspending with cut pipette tips for 15 times on ice. Un-lysed spheroplasts were removed by centrifugation for 10 min at 500 g.

2.2.2.5 Membrane association

All steps were carried out on ice or at 4°C. The spheroplasts were split in 250 µl aliquots and were mixed with 250 µl PS200 buffer, or 250 µl PS200 buffer containing either 2 M KAc, 0.2 M Na_2CO_3, 5 M Urea or SDS (1 % w/v). Samples were incubated for 30 min on ice, except SDS treated sample was incubated at RT. Then high-speed centrifugation at 100,000 x g was done with Optima™ TLX Ultracentrifuge (Beckman) using a TLA 110 rotor in order to separate the cytosolic fraction from the organelle fraction. The supernatant was transferred to a new tube as cytosolic fraction. The pellet was resuspended in 60 µl Urea loading buffer (P). The supernatant was incubated with 1/10 volume of 110% TCA and precipitated (13000 rpm, 4°C, 10 min). The pellet was washed once with 500 µl of ice-cold acetone, centrifuged again and resuspended in 60 µl of urea buffer (S). Samples were incubated for 10 min at 65°C in a heating block with occasional agitation and subjected to SDS-PAGE, followed by immunodetection.

2.2.2.6 Solubility assay

Cells expressing ΔssCG* were grown at 30℃ and shifted to 37℃ for 60 min prior to assay. 20 OD600 of yeast cells were harvested, washed once with 4 volumes of washing buffer and resuspended in 1ml of ice-cold sorbitol buffer (0.7 M sorbitol, 50 mM Tris-HCl pH 7.5, 1 mM PMSF, 1 µg/ml pepstatin-A). Subsequently, all material was kept on ice and cells were lysed with glass beads in ice cold sorbitol lysis buffer. Lysates were pre-cleared by centrifugation at 500 x g for 5 min at 4℃. Total protein (T) was

precipitated from 400 µl of lysate with TCA (11% final concentration). Total protein (T) was solubilized with 60 µl of urea buffer (40 mM Tris-HCl pH 6.8, 8 M Urea, 5% SDS, 100 mM EDTA pH 8, 200 µg/ml Bromophenol blue, 1.5% beta mercapto-ethanol). In addition, 400 µl of lysate was sedimented in a Beckman T110 rotor at 130,000 x g for 30 min at 4℃. The supernatant was subjected to TCA precipitation and treated as soluble protein (S). The pellet of the 130,000 x g centrifugation step was washed once with sorbitol lysis buffer followed by solubilization with 60 µl of urea buffer as described above. Equal amounts of solubilized protein were analyzed by SDS-PAGE followed by immunoblotting. Immunoblots were analyzed with anti CPY or anti PGK antibodies.

2.2.2.7 Fluorescence microscopy in living cells

Cells over-expressing ΔssCPY*-GFP or harboring an empty plasmid were grown at 30℃ and shifted to 37℃ for 60 min prior to viewing fluorescence in living cells. Cells were collected by centrifugation and washed once and resuspended in fresh SC medium. 2.2 µl of suspension was dropped onto a 76 x 26-mm microscopy slide, covered with a coverslip, and subjected to immediate viewing. Fluorescence microscopy was performed with an Axioplan microscope equipped with a 100 X oil-immersion objective (Carl Zeiss) and GFP filter.

2.2.2.8 Immuno-Fluorescence microscopy

1mL of the logarithmic cell culture was fixed by adding 125µL Potassium phosphate buffer (1M, pH 6.5) and 125µL of 37% formaldehyde solution and rotated for 1 hour at RT on a rotator. Cells were later centrifuged at 1000 rpm for 5 min and washed three

times with SP buffer (1.2M Sorbitol, 100mM Potassium phosphate, pH 6.5). Spheroplasts of these cells were made by re-suspending them in 1mL of SP buffer containing 20mM β-ME and 10µL Zymolyase 100T (15mg/ml) and incubating at 30°C for 30 min. The spheroplasts were washed three times with SP buffer and 10µL were added to each well of a diagnostic slide (Serolab, Aidenbach), which was previously coated with Poly-L-Lysine. Spheroplasts were allowed to settle in the wells for 15 min, washed three times with 20µL PBS buffer (53mM Na2HPO4, 13mM NaH2PO4, 75mM NaCl) and incubated for 5 min with 20µL PBT (1% BSA, 0.1% (w/v) Triton X-100 in PBS). Later 20µL of suitably diluted primary antibody was added and incubated in a humid chamber for 2 hours. Subsequently, the samples were washed with PBT and incubated for a further 90 min with secondary antibody. Finally, these were washed with PBS and 2µL mounting solution (80% Glycerol, 0.025µg/ml DAPI, 0.1% p-Phenlynediamine in PBS) was added and later covered the slide with a cover slip. Fluorescence microscopy was performed with an Axioplan microscope equipped with a 100 X oil-immersion objective (Carl Zeiss).

2.2.2.9 Detection of ubiquitin modification

2.2.2.9.1 Detection of ubiquitinated proteins in ER membrane

50 OD_{600} of logarithmically growing yeast cells (OD_{600}1-1.3) were harvested and washed once with 5ml ice-cold washing buffer, once with 2ml ice cold sorbitol buffer and the pellet was resuspended in 0.5ml of sorbitol buffer with 2/3 volume of glass beads. Cells were spheroplasted for 5 pulses of 0.5 min duration in a Mini-bead beater, with cooling on ice between pulses. 1ml of sorbitol buffer was added to samples and pre-cleared by slow centrifugation for 5min at 500g. Pre-cleared spheroplasts were

transferred to Beckman polycarbonated centrifuge tubes (11 x 34 mm) and separated into microsomal pellet and the soluble cytoplasmic fractions by ultracentrifugation at 100,000g for 30min in Beckman optima TLA Ultracentrifuge with a TLA120.2 rotor. The supernatant was transferred to a new tube and Tris-HCl pH7.5, Triton X 100, EDTA, and NaCl were added to final concentration of 50 mM, 1.25%, 6 mM, 190 mM respectively. The pellet was washed with ice cold sorbitol buffer again and solubilized in 100µl of membrane solubilization buffer. 900µl of IP buffer was added and the samples were spun at 13,000rpm for 10 min to remove the insoluble material. The antibody of interest was added both to pellet and supernatant fractions (5µl of monoclonal anti-GFP antibody was used in this study) and IP was carried out for overnight at 4°C. 80µl of Protein-A-Sepharose (7% of Protein-A-Sepharose dissolved in IP buffer w/o Triton X-100) was added to samples and rotated for 2hr, later beads were precipitated by spinning samples at 2, 000rpm for 30sec, washed with 1ml of IP buffer for 5 times and the proteins were denatured by the addition of urea buffer and samples were heated at 65°C for 10 min, 10% SDS-PAGE gels were used and Western blotting and protein transfer were done as described above. Nitrocellulose membranes were autoclaved for 20 min after blotting to enhance the signal. Immunodetection was done as described above.

2.2.2.9.2 Detection of ubiquitinated protein in cytosol

50 OD_{600} of yeast cells over-expressing ΔssCPY*-GFP or harboring an empty plasmid were grown at 25℃ and shifted to 37℃ for 60 min prior to analysis. Cells were washed once with ice-cold washing buffer (20 mM sodium azide, 2 mM PMSF, 20 mM NEM) and resuspended in 500 µl of ice cold IP buffer (50 mM Tris-HCl pH 7.5, 190 mM NaCl,

1.25% TritonX-100, 6 mM EDTA, 2 mM PMSF, 20 mM NEM) and 500 µl of 0.5-mm glass beads were added. Cells were lysed by 5 pulses of 1min duration in a Mini-bead beater, with cooling on ice between pulses. Lysates were pre-cleared by slow centrifugation for 5min at 500g. 1ml of ice cold IP buffer was added to pre-cleared spheroplast and transferred to Beckman polycarbonated centrifuge tubes (11 x 34 mm) and separated into microsomal pellet and the soluble cytoplasmic fractions by ultracentrifugation at 100,000g for 30min in Beckman optima TLA Ultracentrifuge with a TLA120.2 rotor. The supernatant was subjected to immunoprecipitation by anti GFP, fractionated and analyzed using anti ubiquitin or anti CPY antibodies.

2.2.2.10 Pulse-Chase

Growth and Pulse-Chase

Main cultures were prepared by dilution of stationary pre-cultures to OD_{600} =0.3 in selective CM medium. Cells were grown to an OD_{600} of 1.0. 10 OD_{600} were harvested by centrifugation (3000 rpm, 5 min) in a 50 ml Falcon tube and washed three times in 2 ml labeling medium. Cells were resuspended in 1 ml of labeling medium and incubated for 50 min at 30°C. For the pulse 25 µl of [α^{-35}S]-L-Methionine stock solution (about 25 µCi per OD_{600}) were added to the cells and incubated for 20 min. Addition of 1 ml pre-warmed chase medium to the cultures started the chase. Cultures were mixed briefly. 450 µl were removed immediately and added to 50 µl of TCA (110%) in screw capped Eppendorf tubes. Further samples were taken after 30, 60 and 90 min. Samples were stored on ice for subsequent lysis of the cells.

Lysis

Samples were spun down for 8 min at 14000 rpm and the supernatant was removed. Pellet was washed with 1 ml of acetone (-20°C) and spun down for 5 min at 14000 rpm. Supernatant was removed. 100 µl breaking buffer and 2/3 volume of glass beads were added. Samples were placed for 6 times alternately at 95°C for 1 min and vortexed in a multivortexer for 1 min. Samples were stored at –80°C.

Immunoprecipitation

Samples were thawed at 35°C. Protease inhibitor cocktail (Boehringer) was prepared by solving one tablet in 2 ml of ddH_20 and 10 µl of the solution was added to each sample. 1 ml IP buffer was added and samples were vortexed then centrifuged for 15 min at 14000 rpm. 950 µl of the supernatant was transferred to safe-lock Eppendorf tube containing 5 µl of rabbit anti CPY antibody and rotated for 1 hour at RT. Subsequently 80 µl Protein A Sepharose solution (7% Protein A-Sepharose in IP buffer w/o Triton-X-100) was added and samples were rotated for another hour. Samples were washed for three times with 1 ml IP buffer. For elution 60 µl Urea buffer was added and samples were heated at 95°C for 3 min and then centrifuged for 2 min. 15 µl of each sample were loaded on a 10% SDS polyacrylamide gel together with 5 µl of protein marker. Electrophoresis was carried out like described before. Detection was carried out by autoradiography using X-ray film (KODAK Biomax MR Film) and, subsequently, a Storage Phosphor Screen and a PhosphormImager™. Data were analysed using ImageQuant™ software (version 5.2).

3. Results

3.1 Endoplasmic reticulum quality control and degradation (ERQD)

Several studies have revealed that degradation of soluble and membrane bound ERAD substrates involve different components of the ERAD machinery (Kostova and Wolf, 2002). To study these different degradation mechanisms used by ERAD substrates, a set of misfolded proteins sharing CPY* as the degradation motif were generated (Taxis, 2002). One of the model proteins used to study ER quality control in yeast is a mutant form of carboxypeptidase yscY (G255R), commonly known as CPY* (Heinemeyer *et al.*, 1991; Finger *et al.*, 1993; Knop *et al.*, 1993; Hiller *et al.*, 1996; Plemper *et al.*, 1997).

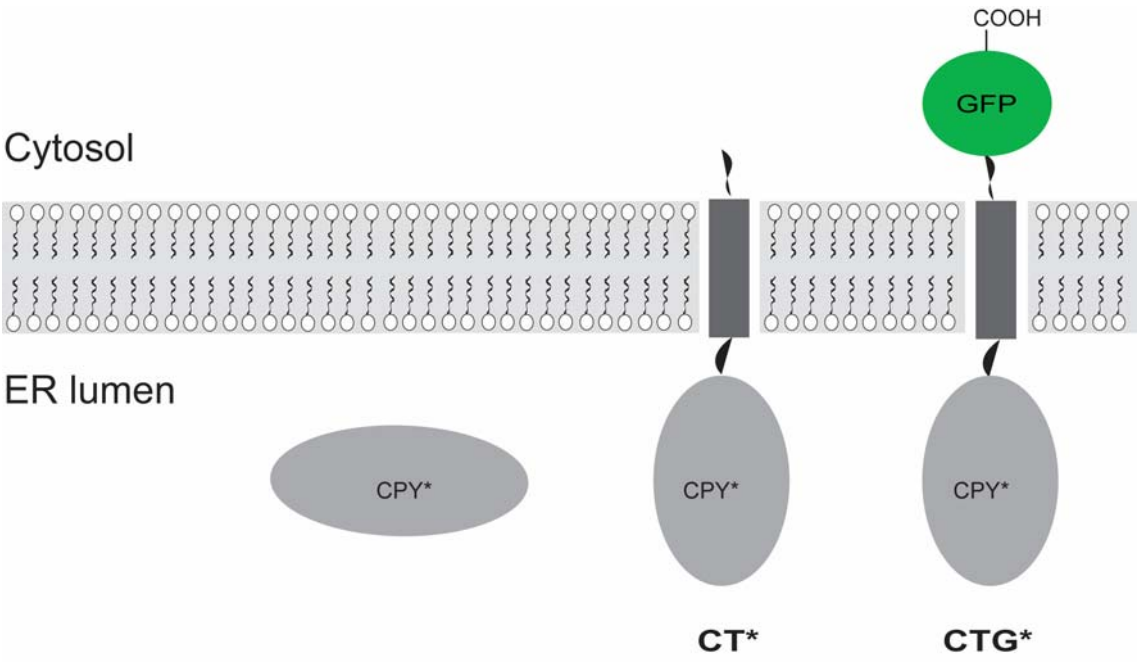

Figure 4. **Schematic drawing of ERAD substrates CPY*, CT* and CTG***

This misfolded protein is translocated into the ER lumen and fully glycosylated but is not transported to the vacuole. Of the two substrates generated based on the misfolded ER lumenal protein CPY*, the first is CT*, in which CPY* is bound to the ER membrane via a single transmembrane domain. In the second (CTG*), the green fluorescent protein (GFP) is fused to CT* providing a cytoplasmic domain (Taxis, 2002). Schematic drawings of these proteins are shown in Figure 4.

In a previous study, Taxis (2002) demonstrated that degradation of CTG* and CT* share the same basic ERAD machinery as CPY*: the elimination process is carried out by the ubiquitin conjugating enzymes Ubc1p and Ubc7p and the ubiquitin-protein-ligase Der3/Hrd1p. It is suggested that Der1p, involved in degradation of soluble CPY* and PrA* (Knop *et al.*, 1996a), is generally involved in the turnover of soluble ERAD substrates, and not in specific recognition of soluble misfolded CPY* only. The breakdown of soluble CPY* or of membrane bound CT* was independent of cytosolic Hsp70 chaperones of the Ssa-family. In contrast, the degradation of CTG* was strongly dependent on the Ssa-family activity along with three J domain proteins, Cwc23p, Jid1p and Hlj1p. Absence of these proteins led to a small but consistent effect on CTG* degradation (Taxis, 2002).

3.1.1 Misfolded integral membrane proteins of the ER, CTG* and CT*, are degraded by the proteasome

We characterized the localization and topology of the fusion proteins CT* and CTG*. Membrane insertion of CT* and CTG* was determined with membrane association experiments. Crude cell extracts were treated with urea, potassium acetate or sodium

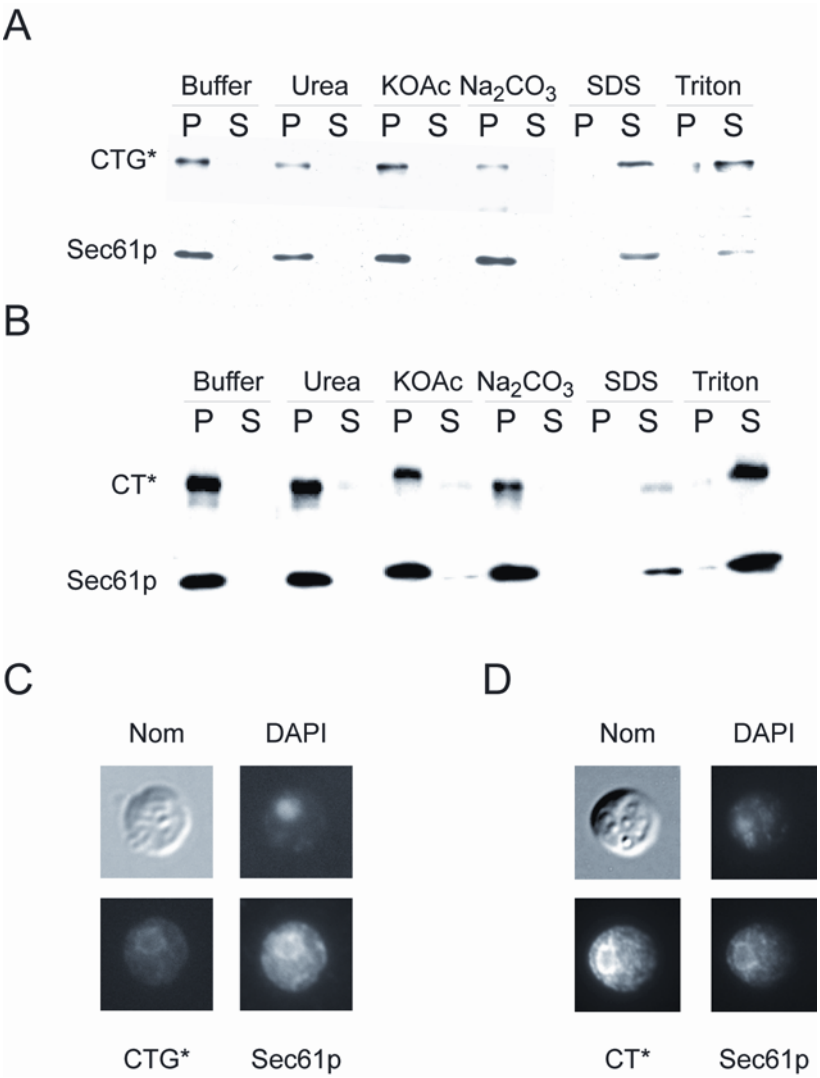

Figure 5. **CTG* and CT* are integral membrane protein of the ER**. Crude extracts from WT (*W303-1C)* cells expressing CTG* (A, Taxics, 2002) and CT* (B) were treated with buffer, or with buffer containing either 2.5 M urea, 0.8 M potassium acetate, 0.1 M sodium carbonate pH 11.6, 1 % SDS or 1 % Triton X-100 followed by centrifugation at 20,000xg. Soluble (S) and pellet (P) fractions were analysed by immunoblotting using anti CPY and anti Sec61 antibodies. Co-localization analysis of CTG* (C), CT* (D) and the ER-membrane protein Sec61p were performed in *W303⊿C* cells. GFP fluorescence shows CTG* localization. Sec61p is visualized by indirect immunofluorescence using anti Sec61 primary and Cy3-conjugated secondary antibody (Nom: Normarski optics, 4', 6'-diamindino-2-phenylindole (DAPI): nuclear staining). CT* was visualized using an anti CPY/Alexa-conjucated anti mouse and Sec61 using an anti Sec61/FITC (fluorescein isothiocyanate)-conjugated anti rabbit antibody sandwich.

carbonate, all known to remove peripheral membrane proteins. CT* and CTG* could only be solubilized after treatment with detergents like Triton X-100 or SDS (Fig. 5A and B). Furthermore, immuno-fluorescence microscopy showed typical ER staining for CTG* and CT* (Fig. 5C and D). In summary, these experiments show that CTG* and CT* are integral type I membrane proteins with their glycosylated N-terminal CPY*-moiety (Taxis, 2002) located in the ER lumen and their C-terminus in the cytosol (Fig. 5).

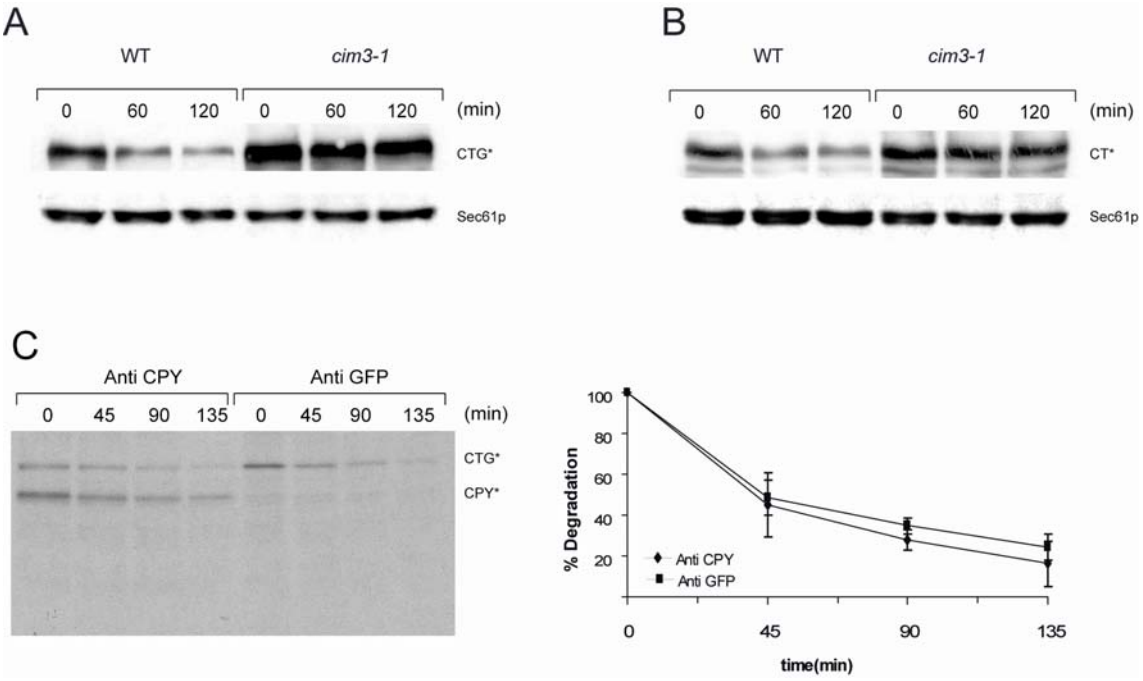

Figure 6. **CTG* and CT* are degraded by the proteasome as a single entity.** Cycloheximide decay experiments were performed in WT and proteasome mutant (*cim3-1*) strains. Cycloheximide was added (t=0 min), samples were collected at the indicated time points and subjected to SDS-PAGE, followed by immunoblotting. Immunoblots were analysed with anti CPY and anti Sec61 antibodies. Substrates: A: CTG*; B: CT*. Pulse chase analysis was done in WT expressing CTG*. CTG* was immunoprecipitated with anti CPY or anti GFP, separated by SDS-PAGE and analyzed using a PhosphoImager and ImagerQuaNT^{TM} (Amersham Bioscience). Plotted data represent the mean values of three independent experiments.

Cycloheximide chase experiments showed that degradation of CTG* and CT* is retarded in proteasomal mutants (Fig. 6A and B). As CTG* has two topologically diverse domains, one residing in the ER-lumen and the other in the cytoplasm, we tested whether both domains are degraded simultaneously. Pulse chase and IP analyses using antibodies recognizing either CPY* or GFP showed that both protein domains were degraded with similar kinetics in wild type cells, indicating that the fusion protein is degraded as a single entity (Fig. 6C).

3.1.2 The ubiquitin-ligase Doa10p is not required for degradation of CT* and CTG*

Since CTG* and CT* are still degraded to some extent in Der3/Hrd1p deleted cells (Taxis, 2002), this might be due to the action of an additional E3 enzyme. Therefore, the involvement of the ubiquitin-protein-ligase Ssm4/Doa10p in the degradation of both proteins was tested. Ssm4/Doa10p has been shown previously to be an E3 enzyme necessary for ERAD of Ubc6p and Ste6*p (Swanson *et al.*, 2001). However, the degradation kinetics of CT* and CTG* were not altered in Δ*ssm4/doa10* or in Δ*ssm4/doa10* Δ*der3/hrd1* cells (Fig. 7), indicating that other components may be involved in the degradation process of these proteins**.**

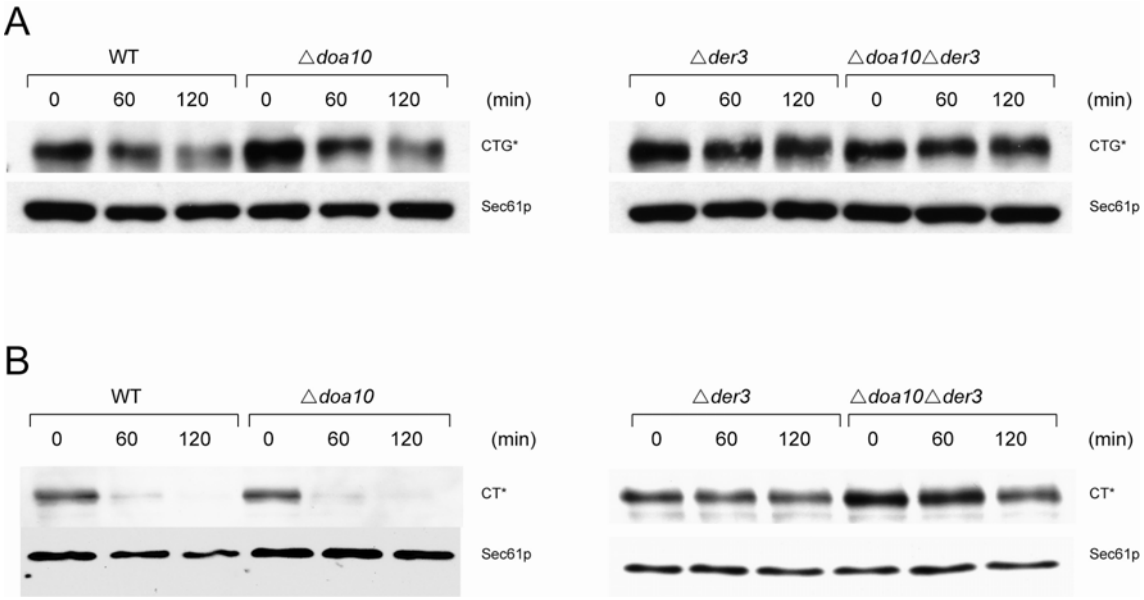

Figure 7. **Degradation of CTG* and CT* do not require Doa10p.** Cycloheximide decay experiments of CTG* (A) and CT* (B) were performed in Δ*doa10*Δ*der3* cells as described in the legend to Figure 6. Sec61p served as a loading control.

3.1.3 The Cdc48p-Ufd1p-Npl4p complex is necessary for the degradation of ERAD substrates

Next, we tested whether the Cdc48p-Ufd1p-Npl4p complex, which was described to be necessary for ERAD of soluble and membrane proteins (Ye *et al.*, 2001; Jarosch *et al.*, 2002; Rabinovich *et al.*, 2002), also acts in the degradation of CTG* and CT*. Cycloheximide chase experiments with *ufd1-1* and temperature sensitive *npl4-1* cells showed that degradation of CTG* and CT* is affected in these mutants (Fig. 8). These findings indicate that action of the trimeric Cdc48p-Ufd1p-Npl4p complex in ERAD is independent of substrate topology.

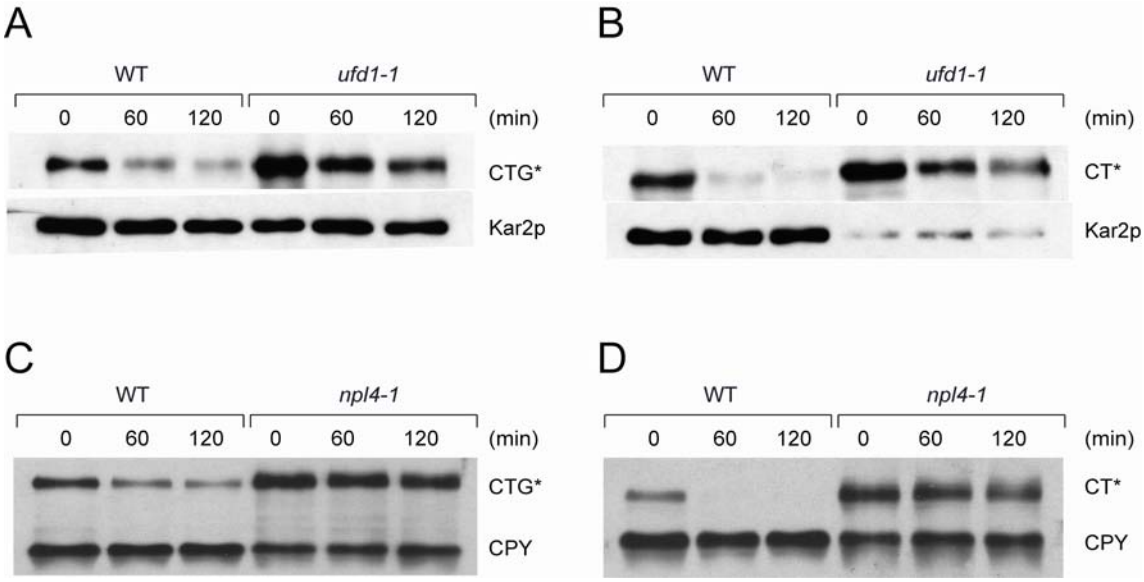

Figure 8. **Degradation of misfolded ER proteins is dependent on the Cdc48-Ufd1-Npl4 complex.** Cycloheximide decay experiments of CTG* (A and C) and CT* (B and D) were performed in *ufd1-1* and *npl4-1* cells as described in the legend to Figure 6. Kar2p and CPY served as loading controls.

3.1.4 Kar2p is only required for degradation of soluble proteins

We were further interested in the role played by the ER lumenal chaperone Kar2p. Kar2p is a member of the highly conserved Hsp70 family, involved in protein import into the ER (Rapoport *et al.*, 1996). Additionally, Kar2p activity is necessary for the degradation of misfolded, soluble proteins like CPY* or mutant α-factor (Plemper *et al.*, 1998; Brodsky *et al.*, 1999b). In contrast, the chaperone is not involved in degradation of polytopic membrane proteins like Pdr5* or CFTR (Plemper *et al.*, 1998; Zhang *et al.*, 2001). Kar2p activity is believed to keep CPY* in a soluble form, to make its dislocation into the cytosol possible (Nishikawa *et al.*, 2001). Additionally, Kar2p may be involved in the recognition of misfolding in CPY*. In this case, degradation of CTG* and CT* should also be dependent on Kar2p. However, in contrast to CPY*, the

degradation kinetics of CTG* and CT* were not changed in the *kar2-159* temperature sensitive cells at restrictive conditions (Fig. 9). No degradation intermediates derived from partially clipped CT* or CTG* proteins were detected. These experiments indicate that Kar2p activity is only important for the degradation of soluble proteins. It is obviously not involved in the recognition of the unfolded state of CPY*.

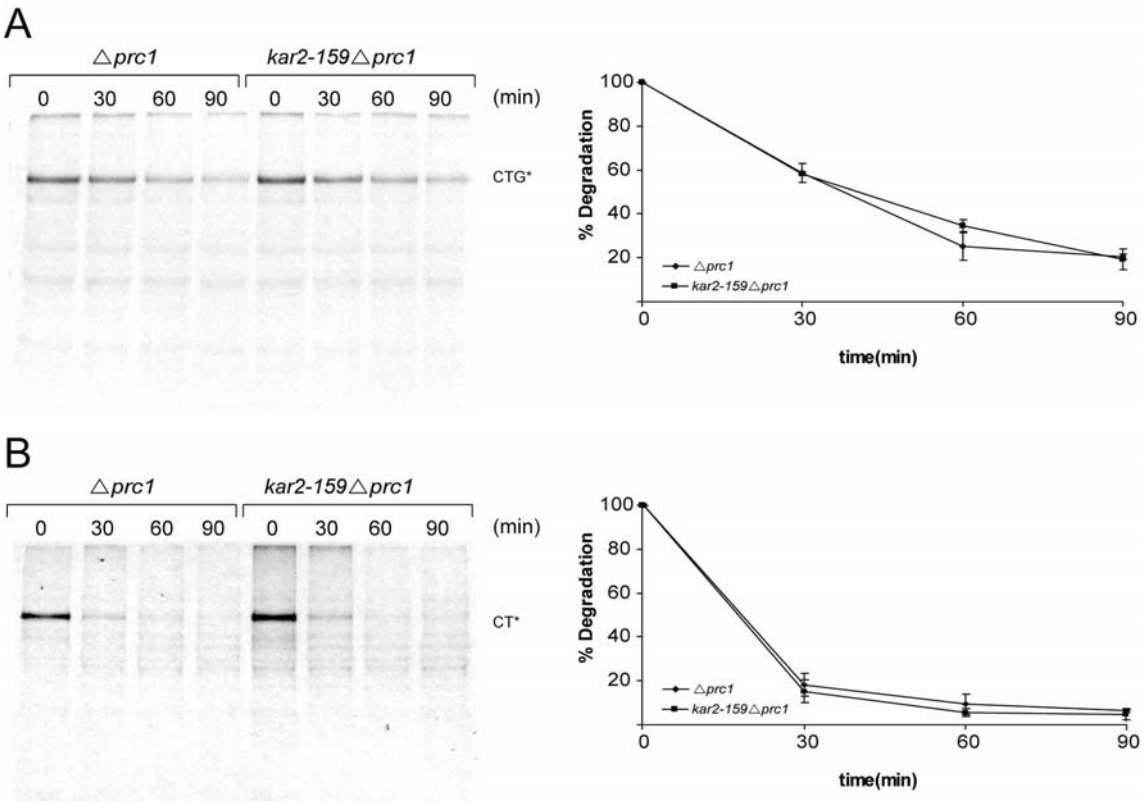

Figure 9. **The ER-lumenal chaperone Kar2p is not necessary for degradation of the membrane proteins CTG* and CT*.** Pulse chase analysis was performed as described in the legend to Fig. 6, except that cells were grown at 25℃ and shifted to 32℃ upon addition of chase media. A and B, degradation of CTG* and CT* in △*prc1* (*W303△C*) and *kar2-159△prc1* mutants, respectively

3.1.5 Hsp104p is required for elimination of CTG* only

Another chaperone known to work together with the cytosolic Hsp70s of the Ssa-family is Hsp104p (Glover and Lindquist, 1998). This protein belongs to the family of Hsp100 chaperones, which are part of the AAA-ATPase superfamily (Neuwald *et al.*, 1999). Hsp100s are known to unfold proteins, either after heat shock (Glover *et al.*, 1998) or prior to hydrolysis (Weber-Ban *et al.*, 1999). Additionally, they bind in an ATP dependent manner to the Ssa1p-Ydj1p complex (Glover *et al.*, 1998). Therefore, we measured the degradation kinetics of CTG* in pulse chase experiments in Δ*hsp104* cells. Interestingly, degradation of CTG* is clearly delayed in Δ*hsp104* cells (Fig. 10). In contrast, degradation of CPY* or CT* is not affected in this mutant (Taxis, 2002). This suggests an additional function of the Hsp70-Hsp40-Hsp104 complex in ERAD of proteins containing a cytosolic domain.

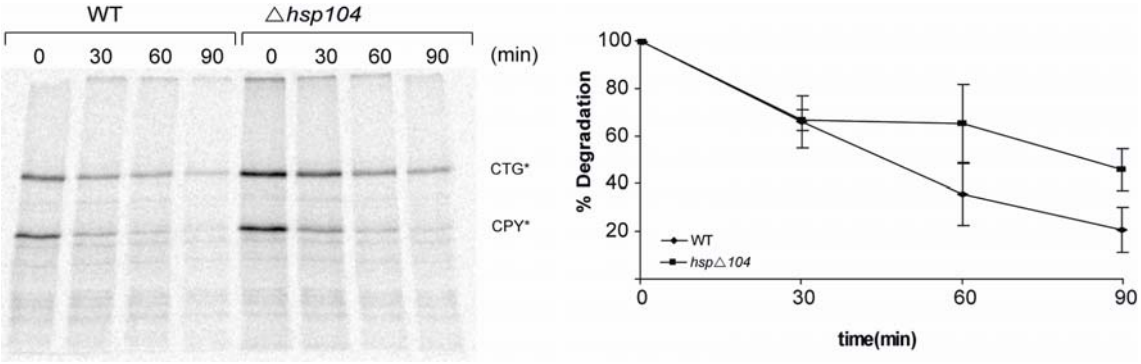

Figure 10. **Hsp104 is involved in the turnover of CTG*.** Pulse chase analysis was performed in WT (*W303-1C*) and △*hsp104* cells.

3.1.6 Ssa1p is required for elimination of CTG* from the ER membrane

Accumulating data show that only CTG*, a misfolded membrane protein with a tightly folded cytoplasmic GFP domain, requires the activity of cytoplasmic Hsp70, Hsp40 and Hsp104 chaperones for proper degradation (Taxis, 2002, Fig. 10). Neither soluble CPY* nor CT* lacking a cytosolic domain require these chaperons for their elimination. Therefore, we tested whether the Hsp70 Ssa1p had an unfolding activity on the tightly folded GFP domain. We analyzed the fluorescence of CTG* in *SSA1* cells and cells carrying the temperature sensitive sss1-45 allele under restrictive conditions after cycloheximide treatment. Prior to addition of cycloheximide, the yeast cell culture was synchronized with hydroxyurea for 3hr. During 3hr of cycloheximide chase, the fluorescence of CTG* dramatically decreased in *SSA1* cells, but was considerably stable in *ssa1-45*^{ts} cells (Fig. 11A). It was known that polyubiquitination of substrates is a prerequisite for delivery of substrates to the proteasome (Ye *et al.*, 2001; Jarosch *et al.*, 2002). In mammalian cells, a CHIP associated Hsp70 chaperone complex facilitates ubiquitination of protein clients and mediates proteasomal degradation (Connell *et al.*, 2001; Demand *et al.*, 2001; Jiang *et al.*, 2001; Murata *et al.*, 2001). Therefore, we addressed the question of whether the cytoplasmic chaperone Ssa1p had a function in polyubiquitination or extraction of CTG* from the ER membrane. To elucidate the step at which Ssa1p may be involved, the localization of polyubiquitinated protein material in *SSA1* and *ssa1-45*^{ts} cells was examined after adding cycloheximide at the restrictive temperature of 37℃ (Fig. 11B). Interestingly, polyubiquitination of CTG* was successfully achieved both in wildtype and mutant strains. Polyubiquitinated CTG* was eliminated from the membrane fraction of *SSA1* cells during 120 min of cycloheximide treatment. Surprisingly, a considerably larger polyubiquitinated CTG*

accumulated in the membrane fraction of *ssa1-45*^{ts} cells after 120 min of cycloheximide treatment. These results may indicate that polyubiquitinated CTG* is not dislocated from the ER membrane in the absence of *SSA1* function even though the ubiquitin-proteasome system is fully functional.

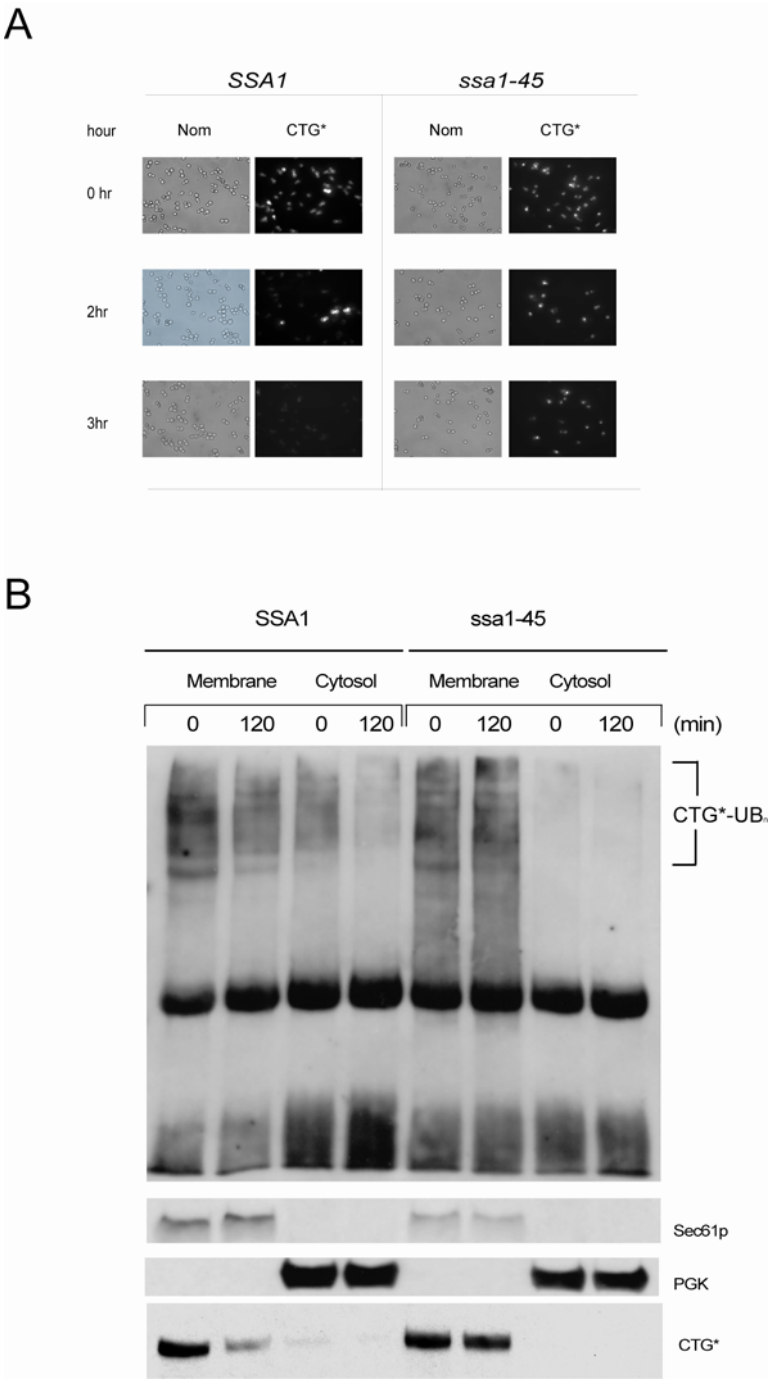

Figure 11. **Polyubiquitinated CTG* is membrane associated in ssa1-45^{ts} cells.** The fluorescence of CTG* was visualized in living cells. Cells expressing CTG* were grown at 25℃ and shifted to 37℃ for 60 min prior to addition of cycloheximide (0.5 mg/ml) to block further protein synthesis. Cells were collected at the indicated time points and subjected to immediate viewing as described in the Material and Methods section. (A). Ubiquitination of CTG* was analyzed Cells expressing ΔssCG* were grown at 25℃ and shifted to 37℃ for 60 min prior to addition of cycloheximide (0.5 mg/ml). The ubiquitination CTG* was assessed in *SSA* and *ssa1-45*^{ts} cells (B). Cells were collected at the indicated time points and spheroplasts were prepared as described in Material and Methods. CTG* was

immunoprecipitated with anti GFP antibody from membrane and cytosol fractions. Before immunoprecipitation one to ten aliquot of the each fraction was subjected to TCA precipitation (11% final concentration) and treated as a control. Immunoblots were analyzed with anti ubiquitin, anti Sec61, anti PGK and anti CPY antibodies.

3.1.7 The chaperone activity of Ssa1p is not restricted to ERAD substrates only

Consequently, we tested another well known proteasomal substrate, Deg1-GFP_2, which is a fusion protein consisting of the Deg1 degradation domain of the MATα2 repressor and undergoes degradation via the ubiquitin-proteasome system (Lenk and Sommer, 2000). It had been previously shown that its elimination is independent of the ERAD delivery pathway to the proteasome (Medicherla *et al.*, 2004).

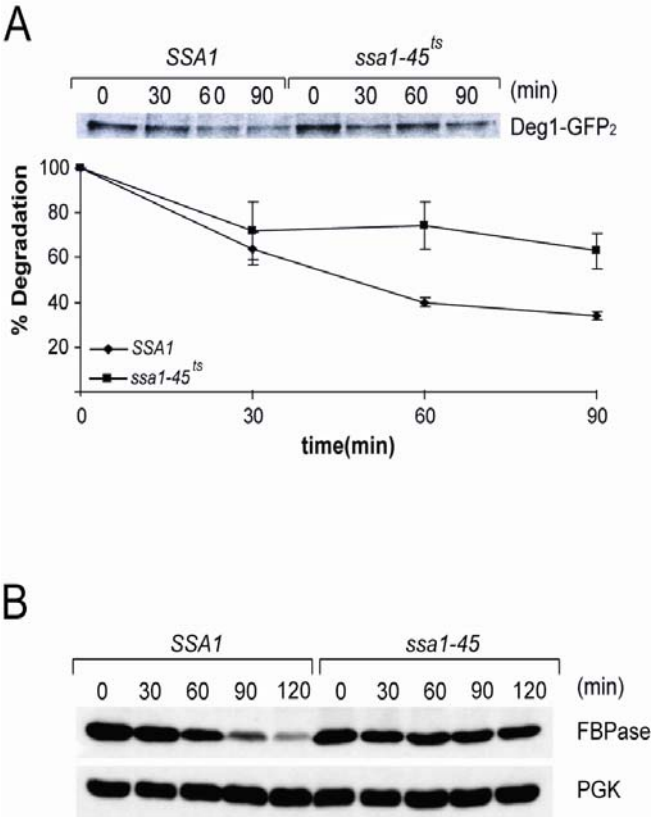

Figure 12. **Elimination of a GFP fusion protein containing a Mat α2 repressor degradation signal and degradation of a metabolic enzyme targeted to the ubiquitin-proteasome system in the cytoplasm requires the chaperone activity of Hsp70.** Pulse chase analysis was done in *SSA1* and *ssa1-45^{ts}* cells expressing Deg1-GFP_2 (A). After 16hr of growth on ethanol medium (YP-Ethanol, 2%), cells were shifted to glucose medium (YPD) and then, samples were collected at the indicated time points and subjected to SDS-PAGE, followed by immunoblotting (B). Immunoblots were analysed with anti FBPase and anti PGK antibodies.

As can be seen in Figure 12A, elimination of the Deg1-GFP_2 fusion protein is considerably delayed in *ssa1-45*^{ts} mutant cells under restrictive conditions. FBPase is the key regulatory gluconeogenetic enzyme which is rapidly inactivated and then degraded in a process called catabolite inactivation (Regelmann *et al.*, 2003). We found the same requirement of chaperones for the proteasomal degradation of this enzyme. After shifting to media containing glucose, degradation of FBPase is considerably delayed in *ssa1-45*^{ts} mutant cells under restrictive conditions (Fig. 12B).

3.2 Molecular chaperones in the cytoplasm quality control (CQD)

We had found that degradation of the misfolded ER protein CTG*, depends on the cytoplasmic Hsp70 chaperone Ssa1p and the cytoplasmic Hsp40's Hlj1p, Cwc23p and Jid1p, as well as the Hsp104 chaperone (Taxis *et al.*, 2003). *In vitro* studies had shown that the 26S proteasome is unable to degrade the GFP moiety of certain fusion proteins probably due to its strongly folded structure (Liu *et al.*, 2003). It was therefore possible that Ssa1p was required for unfolding of the GFP moiety of CTG* to allow its degradation by the proteasome *in vivo*. Since degradation of the cytoplasmically localized substrate △ssCPY*-GFP(△ssCG*) by the proteasome did not require any of the cytoplasmic helper components of the ERAD pathway (Medicherla *et al.*, 2004), we searched for different chaperones which might be involved in its elimination. We reasoned that, as for misfolded ER proteins, recognition, unfolding, escort and delivery machineries may exist to deliver misfolded cytoplasmic proteins to the proteasome for degradation.

3.2.1 The Hsp70 chaperone machinery of Ssa1p is essential for the degradation of cytoplasmically localized misfolded proteins

Previous *in vivo* experiments in yeast had indicated that the Hsp40 cofactor of the Hsp70 chaperone Ssa1, Ydj1p, promotes the degradation of some short-lived and abnormal proteins (Lee *et al.*, 1996), thus suggesting a requirement for Hsp70. We therefore assessed whether the Hsp70 chaperone machinery of the Ssa class had a role in the degradation of △ssCPY*-GFP (△ssCG*). We tested the requirement for the

Hsp70 Ssa chaperones by comparing the properties of two strains, both of which lack three of the four Ssa proteins (Ssa2p, Ssa3p, and Ssa4p). In *ssa1-45^{ts}* cells Ssa1 is present as a temperature sensitive allele, whereas in isogenic *SSA1* cells the gene is present as a wild type copy (Becker *et al.*, 1996; Taxis *et al.*, 2003). As seen in Figure 13A, degradation of ΔssCG* progresses with a half life of 20-30 minutes in *SSA1* cells. Degradation of △ssCG* is nearly completely abolished in *ssa1-45^{ts}* cells under restrictive conditions. A similar dependence on Ssa1 for ΔssCG* degradation is observed using antibodies directed against either CPY or GFP for immunoprecipitation (Fig. 13A). As expected, degradation of endogenously expressed CPY*, which is retrotranslocated from the ER lumen to the cytoplasm (Hiller *et al.*, 1996), is not affected by the absence of Ssa1p (Taxis, 2002). To test whether the position of the strongly folded GFP domain within ΔssCG* had any effect on the degradation and whether its context influenced the Ssa1p-dependence of degradation, we constructed ΔssGFP-CPY* (ΔssGC*), carrying GFP N-terminally fused to signal sequence deleted CPY*. As seen in Figure 13B, ΔssGC* is degraded nearly as rapidly as ΔssCG* and lack of an active Ssa blocks degradation of this substrate as well. Also, fusion of a variant GFP_{uv} that fluoresces more brightly than wild type GFP at the C-terminus of ΔssCPY* does not affect the half life of Δss$CG*_{uv}$ degradation (Fig. 13C).

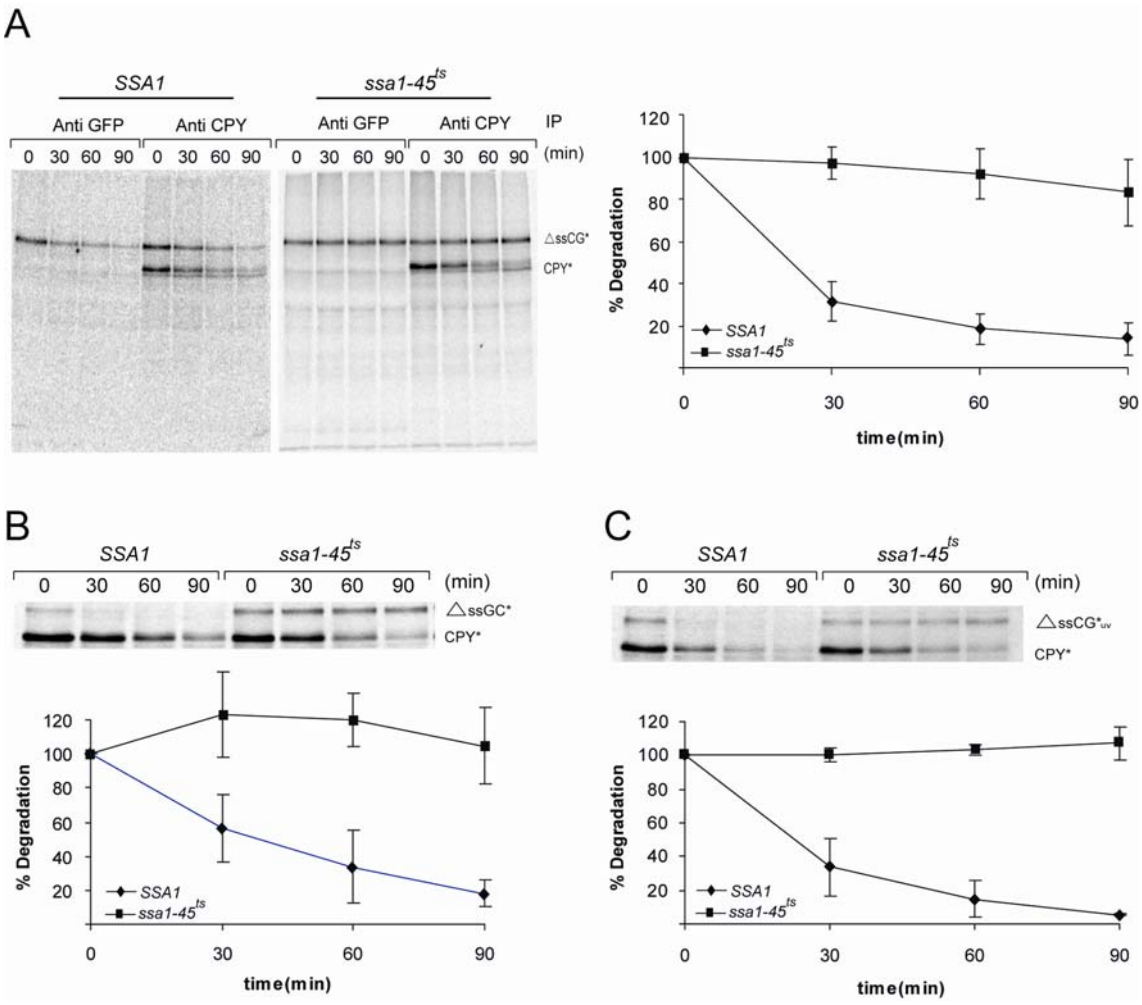

Figure 13. **The Hsp70 chaperone machinery of Ssa1p is required for the degradation of cytoplasmically localized misfolded ΔssCPY*-GFP fusion proteins.** Pulse chase analysis was done in *SSA1* and *ssa1-45*^{ts} cells. Cell extracts were immunoprecipitated with anti CPY (A, B and C) or anti GFP (A). Substrates: A: ΔssCG*; B: ΔssGC*; C: ΔssCG*$_{uv}$. The ERQD substrate CPY* served as a control.

3.2.2 Hsp70 species has a more general function in the degradation of cytoplasmically located misfolded proteins

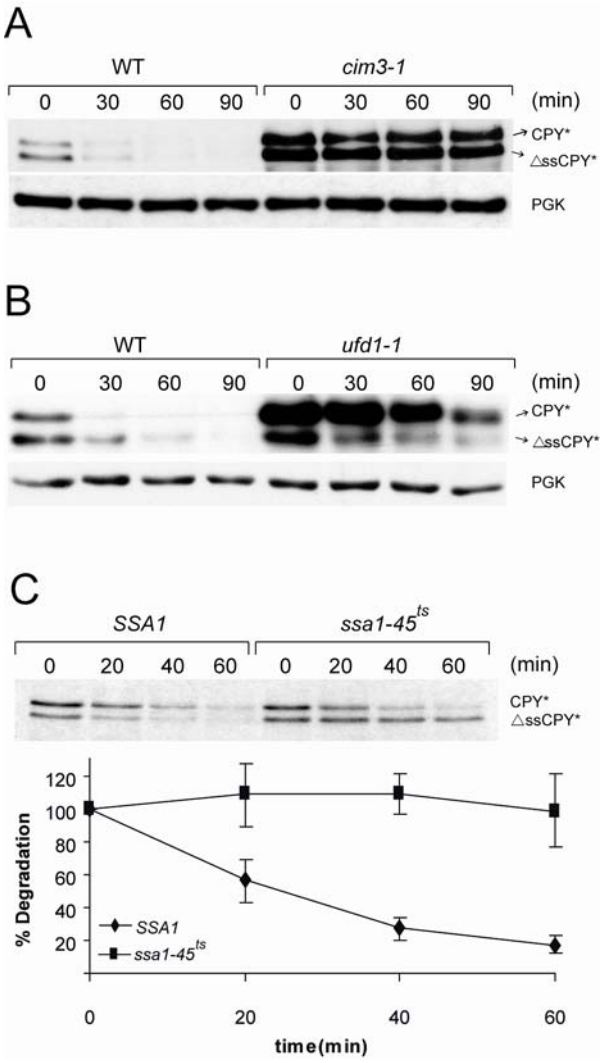

Figure 14. **Degradation of misfolded and ER import incompetent CPY* is dependent on the proteasome and Ssa1p but not on the Cdc48-Ufd1-Npl4 complex.** Cycloheximide decay experiments were performed in the proteasomal mutant *cim3-1* (A) and in *ufd1-1* cells (B) expressing ΔssCPY*. Immunoblots were analyzed with anti CPY and anti PGK as a loading control. Pulse chase analysis in *SSA1* and *ssa1-45*^{ts} cells (C) was performed and analyzed as described in the legend to Fig. 6. The ERQD substrate CPY* served as a control.

As mentioned before, *in vitro* studies had shown that the 26S proteasome is unable to degrade the GFP moiety of certain fusion proteins, due to its strongly folded structure (Liu *et al.*, 2003). It was therefore possible that Ssa1p was only required for unfolding of the GFP moiety of ΔssCPY*-GFP (ΔssCG*) to allow its degradation by the proteasome *in vivo.* To explore this possibility, another misfolded CPY* variant was constructed by Z. Kostova of our laboratory. This variant, called ΔssCPY*, lacks its signal sequence, preventing it from entering the ER and is therefore located in the cytosol (F. Eisele of our laboratory). This protein is rapidly degraded by the proteasome: elimination of ΔssCPY* is severely disrupted in the proteasome mutant *cim3-1* (Fig. 14A). It has been previously shown (Medicherla *et al.*, 2004) that the elimination of cytosolic ΔssCPY*-GFP does not require the trimeric Cdc48p-Ufd1p-Npl4p complex. Testing for degradation in *ufd1-1* mutant cells shows that Cdc48p-Ufd1p-Npl4p is not involved in the proteasomal elimination of ΔssCPY* either (Fig. 14B). As shown in Figure 14C, ΔssCPY* is rapidly degraded in *SSA1* but, surprisingly not in *ssa1-45*^{ts} mutant cells under restrictive conditions. These experiments indicate that the Ssa machinery is generally needed for the degradation of misfolded proteins of the cytoplasm.

3.2.3 Ssa1p seems to function in the recognition of the misfolded ΔssCPY* domain of the fusion protein

To further assess that degradation of the GFP domain by the proteasome is independent of Ssa proteins, we tested the degradation of GFP linked to the C-terminal 37 amino acids of mouse ornithine decarboxylase (cODC). This 37 amino acid C-

terminal sequence is a transferable element with the capacity to direct diverse proteins for ubiquitin-independent proteasomal degradation (Hoyt *et al.*, 2003; Zhang *et al.*, 2003; Zhang and Coffino, 2004).

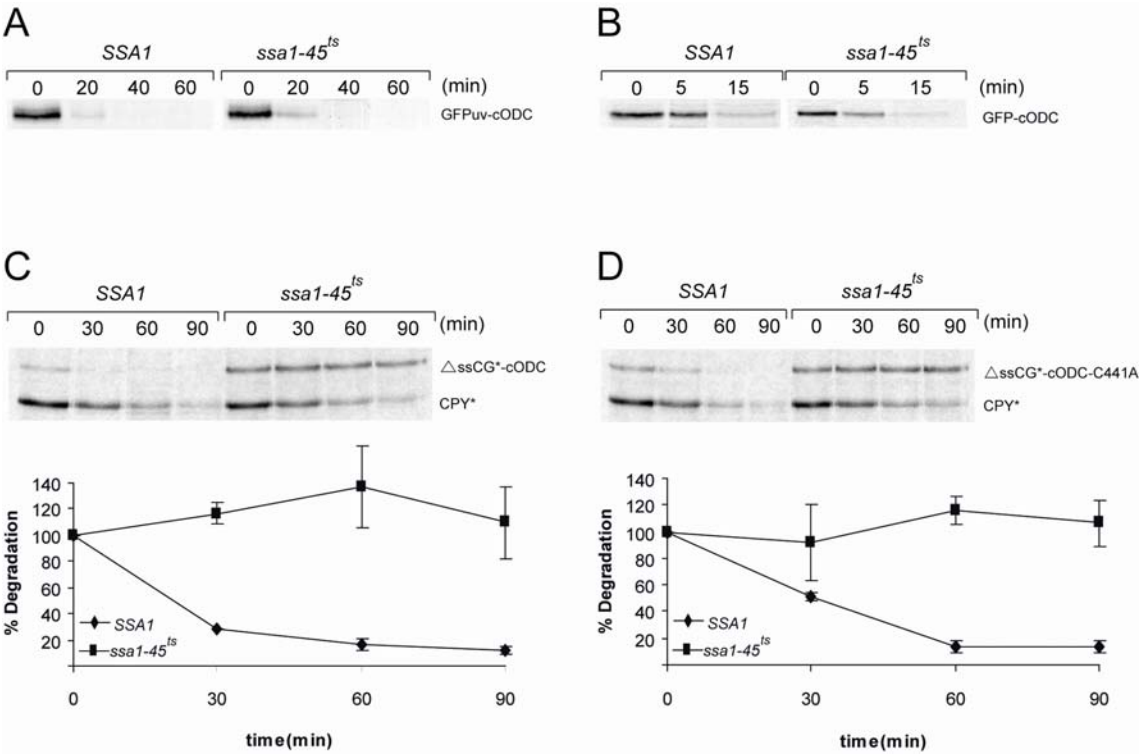

Figure 15. **Ubiquitin independent degradation of GFP-cODC does not require Ssa1p activity, but its fusion to ΔssCPY* makes the process Ssa1p dependent.** Pulse chase analysis was done in *SSA1* and *ssa1-45*^{ts} cells expressing GFP$_{uv}$-cODC (A), GFP-cODC (B), ΔssCG*-cODC (C) and ΔssCG*-cODC-C441A (D).

The Ssa1 dependency of the fusion proteins GFP$_{uv}$-cODC and GFP-cODC (Fig. 15A and B, J. Takeuchi, University of California, San Francisco) was tested. The GFP-cODC proteins are rapidly degraded, regardless of the Ssa status of the cell: turnover is similar in *SSA1* and *ssa1-45*^{ts} mutant cells, whether tested under permissive or

restrictive conditions (Fig. 15A and B). These experiments indicate that, in the cellular environment, there must be means to unfold the GFP domain for degradation that do not depend on the Ssa machinery. Interestingly, degradation of a fusion protein consisting of ΔssCPY* and GFP_{uv}-cODC (ΔssCG*-cODC) became dependent on Ssa1p just like ΔssCG* (Fig. 15C). It has been reported that the C-terminal 37 amino acids of ODC represent a critical signal for rapid ODC degradation and that a mutation of Cys_{441} to Ala_{441} in this sequence causes a significant stabilization of ODC or of proteins to which cODC is attached (Hoyt *et al.*, 2003). Interestingly, the Cys_{441} to Ala_{441} mutation in ΔssCG*-cODC-C441A did not lead to stabilization, but directed this protein to a form of degradation which relied on the Ssa1 protein (Fig. 15D). This finding suggests that the Ssa1p directed degradation of the ΔssCPY* moiety dominates over the Ssa1p-independent cODC-directed degradation in the fusion protein.

3.2.4 The fate of the cytoplasmically mislocalized wild type CPY is similar to its mutated counterpart

Import of secretory proteins into the ER can be faulty (Levine *et al.*, 2005). Since intracellular mislocalization of proteins may lead to cellular dysfunction, we were interested in the question of how wild type secretory proteins which fail to advance into the ER are handled by the cell's cytosol. We chose mislocalized but otherwise wild type carboxypeptidase yscY (CPY) for this analysis. We analyzed the fate of ΔssCPY which lacks a signal sequence. The mislocalized and presumably misfolded ΔssCPY is rapidly degraded; its turnover is performed by the proteasome, as evidenced by the stabilization in the proteasomal *cim3-1* mutant (Fig. 16A). As for the mutated

cytoplasmic located CPY species, degradation of ΔssCPY is independent of the trimeric Cdc48p-Ufd1p-Npl4p complex required for elimination of misfolded ER proteins (Fig. 16B). However, elimination of ΔssCPY does require an intact Ssa1 protein (Fig. 16C). The fate and chaperone dependence of the cytoplasmically mislocalized wild type CPY species is similar to that of its mutated counterpart in the cytoplasmic environment.

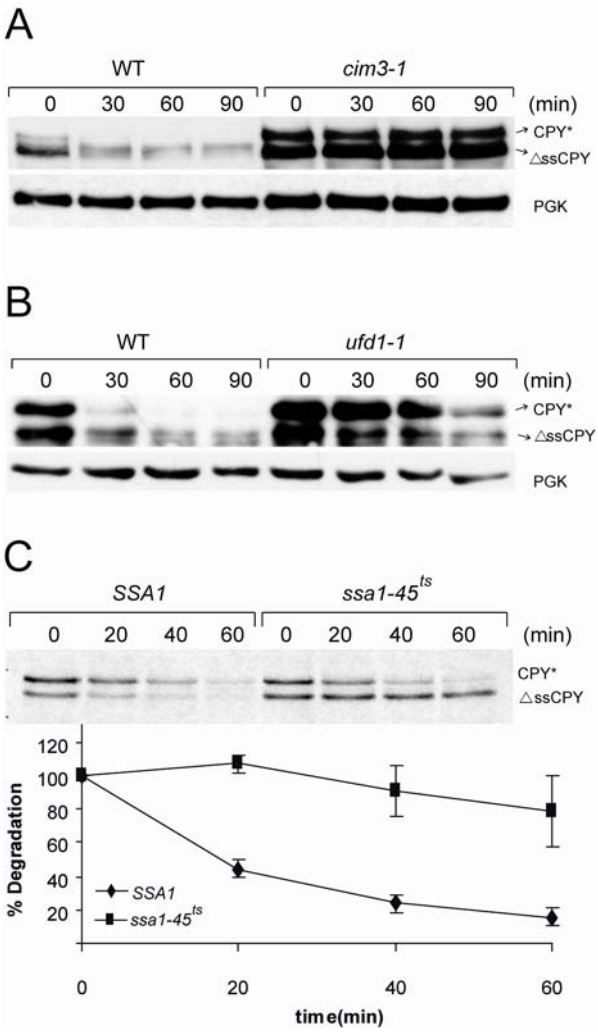

Figure 16. **Degradation of the cytoplasmically mislocalized wild type CPY is similar to its mutated counterpart.** Cycloheximide decay experiments (A and B) and pulse chase analysis (C) were performed as described before.

3.2.5 Mutation of putative ubiquitination sites in Ssa1p does not affect the activity of Ssa1p

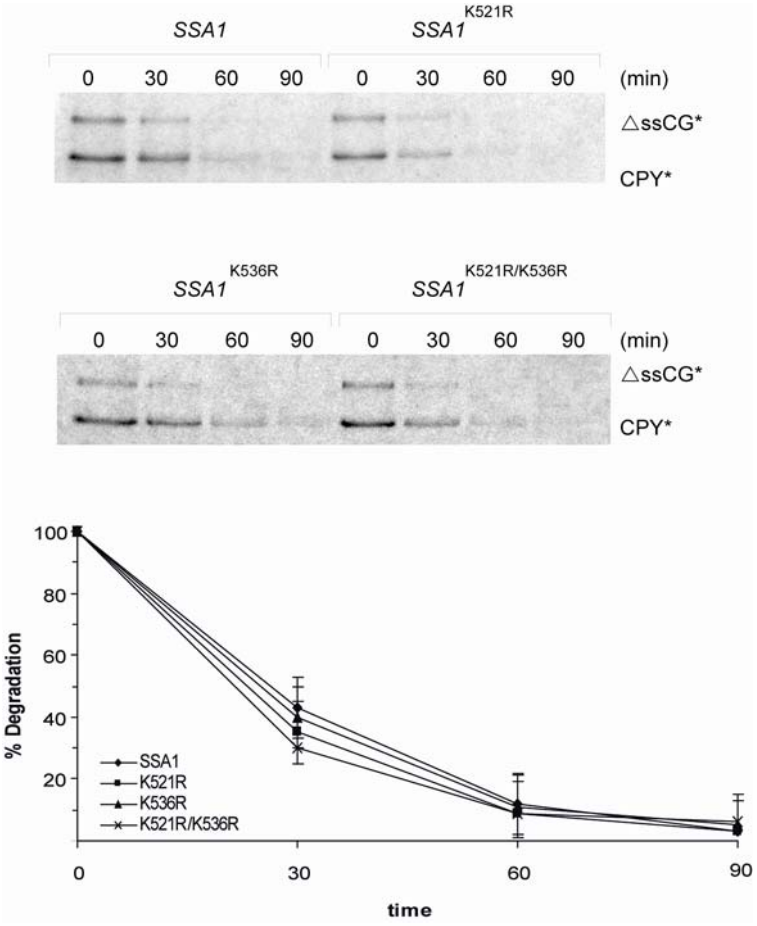

Figure 17. **The blocking of putative ubiquitination sites in Ssa1p does not alter the chaperone activity.** Pulse chase analysis was done in *SSA1* and $SSA1^{K521R}$, $SSA1^{K536R}$ *and* $SSA1^{K521R/K536}$ expressing ΔssCPY*-GFP (ΔssCG*).

In recent studies in mammalian cells, the CHIP protein was identified as an E3 ubiquitin ligase for chaperones (Demand *et al.*, 2001; Jiang *et al.*, 2001; Murata *et al.*, 2001). Interestingly, the molecular chaperone Hsc70 is ubiquitinated by CHIP via non-canonical ubiquitin chains that utilize either lysine 29 or 63 of ubiquitin and that do not

target Hsc70 for proteasome-mediated degradation. This suggests that ubiquitin modification of Hsc70 may alter the function of Hsc70, serve as a targeting sequence, or otherwise alter cellular signaling events. Even though there is no known CHIP homologue in yeast yet, putative ubiquitination sites on *SSA1* were predicted by proteomics analysis (Hitchcock *et al.*, 2003; Peng *et al.*, 2003). Three such mutant alleles of *SSA1*, namely $SSA1^{K521R}$, $SSA1^{K536R}$ *and* $SSA1^{K521R/K536}$ were tested to see whether they influence Ssa1p-dependent degradation. However, no changes in ΔssCG* degradation were observed by blocking the putative ubiquitination sites in Ssa1p (Fig. 17).

3.2.6 The Hsp70 co-chaperone Ydj1p is required for the degradation of cytoplasmically localized misfolded proteins

Hsp70 chaperones function in a complex with co-chaperones of the Hsp40 family, which modulate the substrate specificity of the Hsp70s (Cheetham *et al.*, 1998; Johnson and Craig, 2001; Rudiger *et al.*, 2001; Fan *et al.*, 2003). In a previous study, we had shown that the Hsp40 co-chaperones Hdj1p, Cwc23p and Jid1p are required for degradation of the ERQD substrate CTG* (Taxis *et al.*, 2003). However, none of these Hsp40 co-chaperones are needed for the degradation of cytoplasmic ΔssCG*, (Fig. 18A). In contrast, the Hsp70 co-chaperone Ydj1p has a strong influence on degradation of ΔssCG*, as well as ΔssCPY* and ΔssCPY: degradation of all three cytosolic model substrates is considerably slowed down in $ydj1\text{-}151^{ts}$ mutant cells under restrictive conditions (Fig. 18B-D). Ydj1p is not required for any of the ERQD substrates derived from CPY* (Taxis *et al.*, 2003). It can be concluded, then, that the CQD

(cytoplasmic quality control and degradation) substrates ΔssCG*, ΔssCPY* and ΔssCPY in contrast to the ERQD substrate CTG* have different co-chaperone requirements.

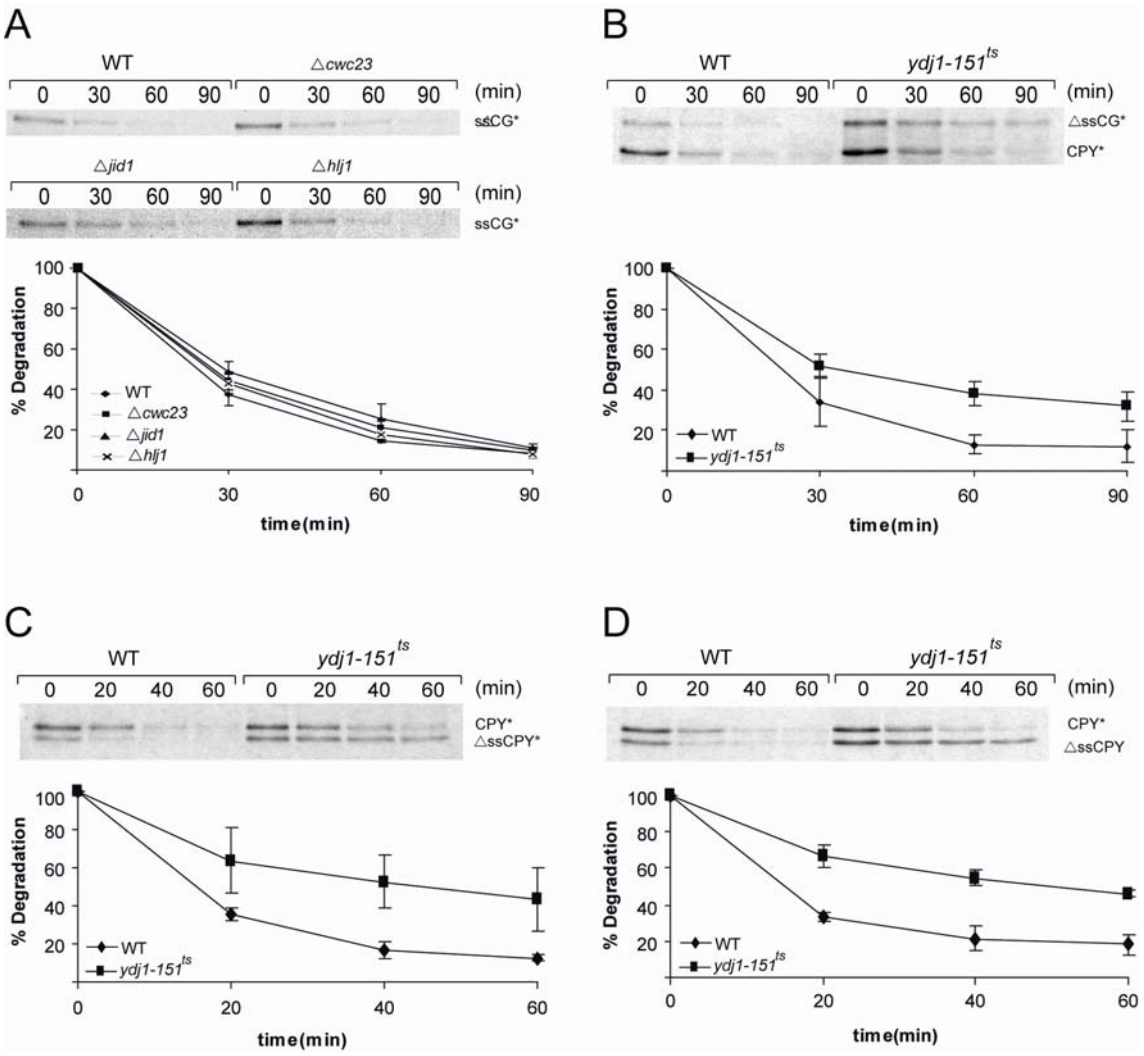

Figure 18. **The Hsp70 co-chaperone Ydj1p promotes the degradation of cytoplasmically localized misfolded proteins.** Pulse chase analysis was performed in wild type (WT), J domain proteins of Hsp40 co-chaperones (A) and *ydj1-151*^{ts} cells expressing ΔssCG* (A and B), ΔssCPY* (C) and ΔssCPY (D).

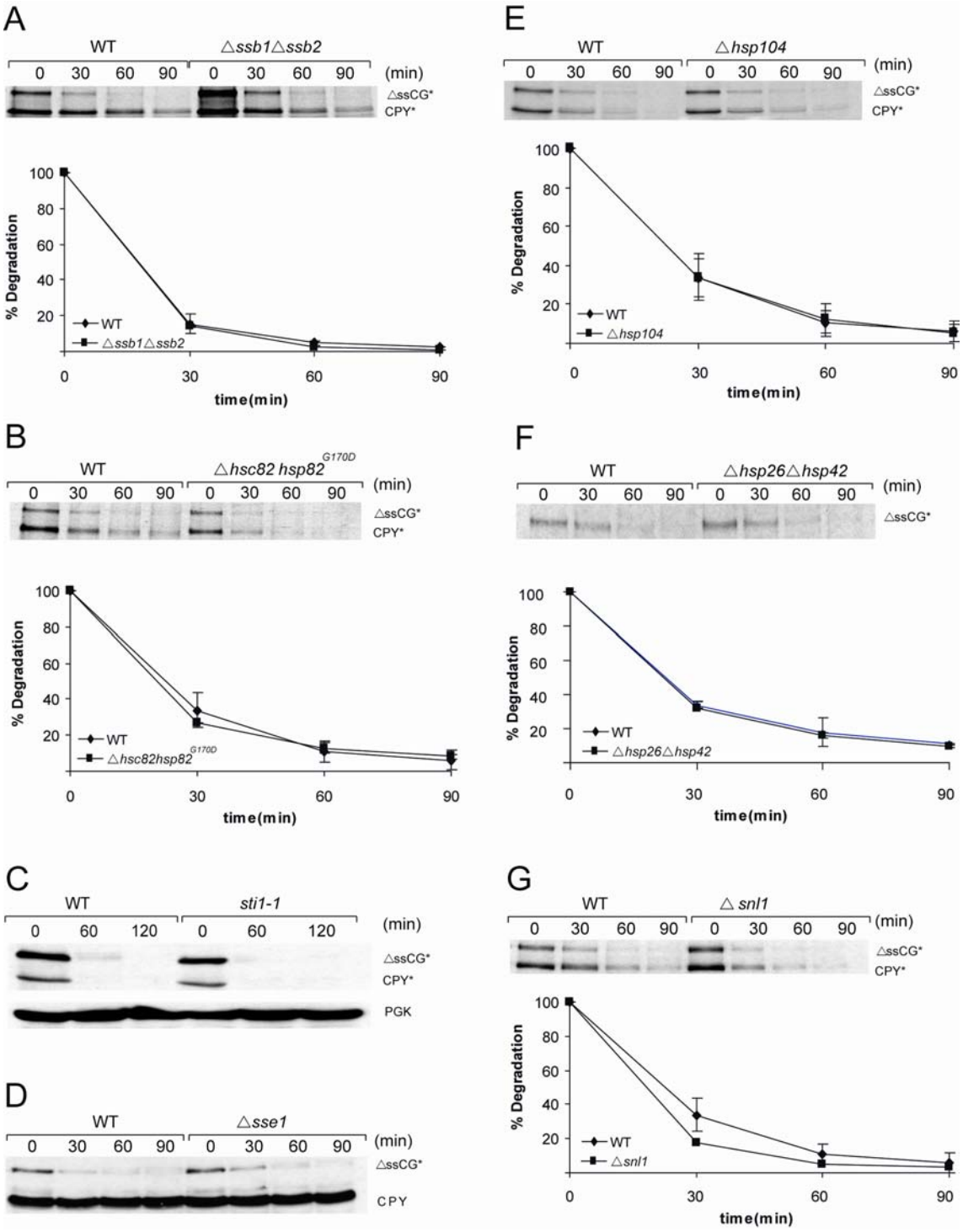

Figure 19. **The Hsp70 Ssb class, the Hsp90 complex, Hsp104, Hsp110, small heat shock proteins Hsp26, Hsp42 and the yeast Bag1 homologue, Snl1p, are not involved in the degradation of ΔssCG*.** Pulse chase analysis was done in Δ*ssb1*Δ*ssb2* (A, Bolender, 2005), Δ*hsc82hsp82*^{G170D} (B), Δ*hsp104* (E),

Δ*hsp26*Δ*hsp42* (F) and Δ*snl1* (G) cells expressing ΔssCG* and cycloheximide decay experiments were performed in *sti1-1* (C, Bolender, 2005) and Δ*sse1* (D, Bolender, 2005) cells expressing ΔssCG*. PGK and CPY were served as loading controls.

3.2.7 Other molecular chaperones are not involved in the degradation of △ssCG*

Another class of Hsp70 chaperones, the Ssb members, are ribosome associated and involved in the folding of newly synthesized polypeptide chains (Pfund *et al.*, 1998; Pfund *et al.*, 2001). We tested a strain defective in this chaperone family (Δ*ssb1*Δ*ssb2*) and found that they are dispensable for degradation of ΔssCG* (Fig. 19A, Bolender, 2005). We also tested whether components of the Hsp90 chaperones were involved in degradation of ΔssCG*. The yeast Hsp90 chaperone family consists of two proteins, Hsc82p and Hsp82p. These are associated with the co-chaperone Sti1p/HOP, which is also an activator of the Ssa1 proteins (Nathan *et al.*, 1997; Wegele *et al.*, 2003). The Hsp90 chaperones Hsc82p and Hsp82p are not required for degradation of ΔssCG* (Fig. 19B). Consequently, the Hsp70/Hsp90 co-chaperone Sti1p/HOP has no effect on the degradation of ΔssCG* (Fig. 19C, Bolender, 2005). It has been suggested that another major cytoplasmic chaperone, Hsp104, works together with the Hsp70s of the Ssa family and binds to the Ssa1p-Ydj1p complex, in an ATP-dependent manner, to unfold proteins (Parsell *et al.*, 1993; Parsell *et al.*, 1994; Glover *et al.*, 1998; Lum *et al.*, 2004). ER associated degradation of CTG* requires both Ssa and Hsp104 chaperones (Taxis *et al.*, 2003). However, Hsp104p is not required for elimination of ΔssCG* (Fig. 19E). We were further interested in the involvement of the Hsp110 chaperone Sse1p in elimination of ΔssCG*. The Sse1p protein is a component of the Hsp90 chaperone

complex and mediates degradation of misfolded VHL (McClellan *et al.*, 2005a). No function of Sse1p in ΔssCG* degradation can be observed (Fig. 19D, Bolender, 2005). Two small heat shock proteins, Hsp26 and Hsp42 are ubiquitous molecular chaperones which protect yeast cells from a variety of cellular stresses. *In vitro* they have been found to bind to unfolded proteins to form large co-complexes and by this prevent their aggregation (Haslbeck *et al.*, 1999; Haslbeck *et al.*, 2004; Cashikar *et al.*, 2005). We tested the involvement of Hsp26 and Hsp42 in degradation of ΔssCG*. As seen in Figure 19F, degradation of ΔssCG* is not affected by the absence of Hsp26 and Hsp42. Recently, in the cytosol of higher eukaryotic cells, BAG domain proteins were shown to interact with Hsp70 chaperones as nucleotide exchange factors. In mammalian cells, together with the E3 ligase CHIP, they are known to be partners in a degradative Hsp70 complex (Esser *et al.*, 2004). The yeast BAG-1 homologue, Snl1p, functionally interacts with Hsp70 chaperones (Sondermann *et al.*, 2001; Sondermann *et al.*, 2002). However, no alteration of degradation of ΔssCG* is seen in *SNL1* deletion mutant cells (Fig. 19G).

3.2.8 Molecular chaperone machinery of Ssa and its co-chaperone Ydj1p are required for rescue of aggregated △ssCG*

We tested whether the Ssa machinery has any function in keeping misfolded ΔssCG* in a soluble state in the cytoplasm. In wild type cells harboring all four Ssa chaperones (Ssa1p, Ssa2p, Ssa3p, Ssa4p) most of the ΔssCG* protein is in soluble state and this does not change when cells are shifted from 30℃ to 37℃ (Fig. 20B).

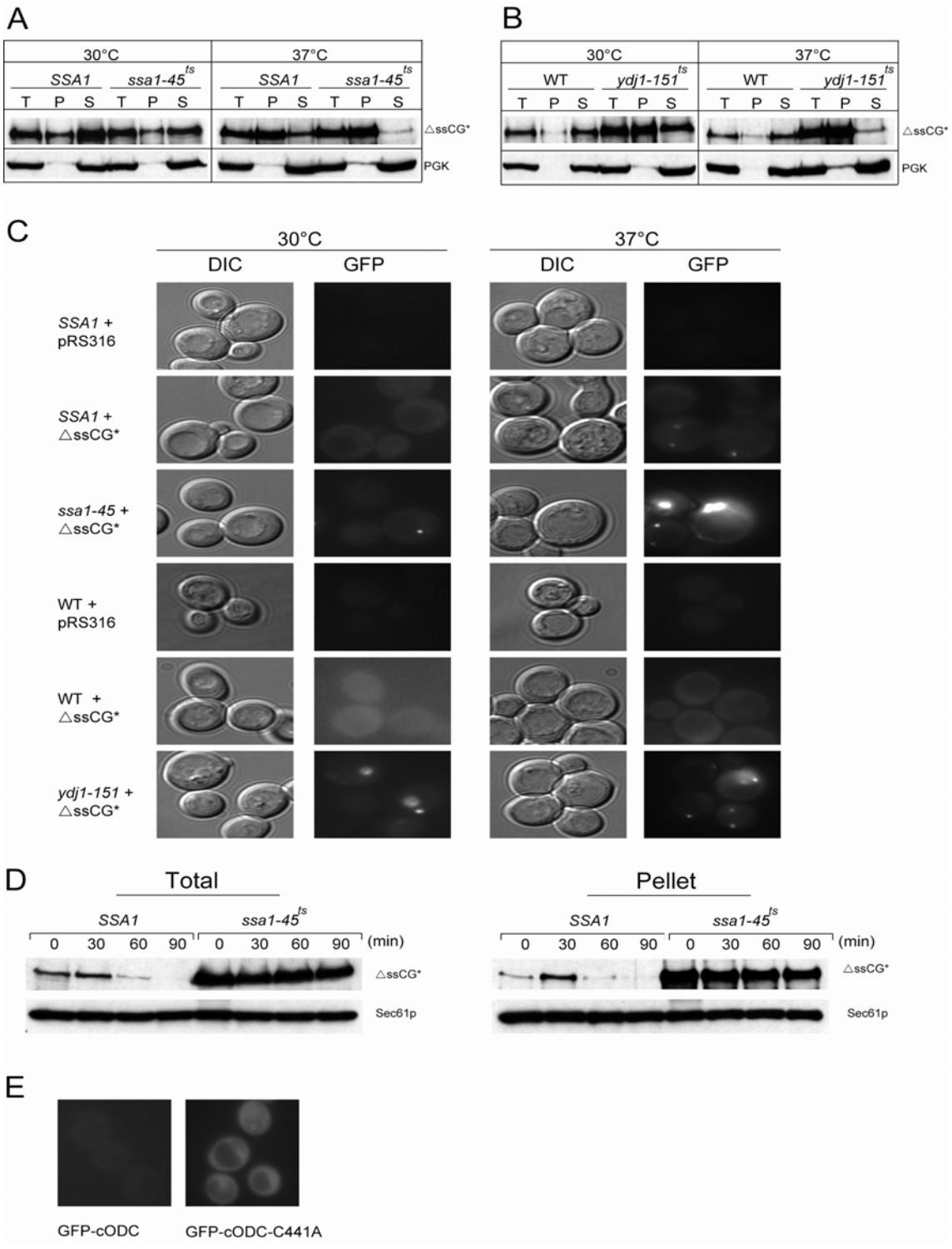

Figure 20. **Ssa1p and its co-chaperone Ydj1p are required for rescue of aggregated ΔssCG*.** Cells expressing ΔssCG* were grown at 30℃ and shifted to 37℃ for 60 min prior to the solubility assay. The solubility of ΔssCG* was assessed in *SSA1, ssa1-45*^{ts} (A), wild type *W303-1C (SSA1, SSA2 ,SSA3, SSA4)*

and *ydj1-151*^{ts} strains (B). The same amount of total (T), supernatant (S) and pellet (P) fraction was analyzed via SDS-PAGE and immunoblotting. Immunoblots were analyzed with CPY antibody and PGK antibody as a control. The fluorescence of ΔssCG* was analyzed in living cells (C) as described in Material and Methods. The cells harboring over-expressed ΔssCG* or an empty plasmid were grown at 30℃ and shifted to 37℃ for 60 min prior to analysis. All cells were visualized by fluorescence microscopy using equal exposure times and conditions. Re-solubilization of aggregated ΔssCG* was assessed in *SSA1* and *ssa1-45*^{ts} cells (D). After temperature shift to 37℃ for 1hr, cycloheximide was added to a final concentration of 0.5 mg/ml to block further protein synthesis. 20 OD_{600} of cells were taken at the indicated time points and treated as indicated for the above solubility assay. Sec61p served as control. Three independent experiments gave similar results. The fluorescence of GFP-cODC and GFP-cODC-C414A were analyzed in *SSA1* cells at 37℃ as stated above (E).

As seen in Fig. 20A, when *SSA1* cells containing only Ssa1p are transferred from 30℃ to 37℃, the ΔssCG* material in the pellet increases, indicating aggregation of the misfolded protein with increased temperature. One interpretation of these findings is that in the absence of Ssa2, Ssa3 and Ssa4, the single Ssa1p protein is functioning at or beyond its limits in keeping misfolded protein soluble under heat stress. However, analysis of the amount of soluble and precipitated cellular protein material may not be fully informative, since it may easily be influenced by the experimental conditions used. We therefore analyzed the solubility of ΔssCG* in the different strains by fluorescence microscopy, visualizing the distribution of the GFP moiety of the protein. As seen in Figure 20C, no precipitated ΔssCG* material can be seen at 37℃ in wild type cells containing all four Ssa species, regardless of whether ΔssCG* was expressed from a single- (data not shown) or multi-copy plasmid (Fig. 20C). In contrast, at 37℃ some punctated fluorescent dots, indicating presence of precipitated material, are visible in cells containing only Ssa1p, substantiating the *in vitro* finding. Nevertheless, the

misfolded protein is rapidly degraded in *SSA1* cells at 37℃ (Fig. 13). A dramatic increase in precipitated fluorescent material appears under the restrictive conditions of 37℃ in the *ssa1-45* and *ydj1-151* mutant cells. Under restrictive conditions in *ssa1-45^{ts}* mutant cells, we see most of the misfolded ΔssCG* material in the pellet (Fig. 20A) and degradation is completely blocked (Fig. 13A and C). The behavior of ΔssCG* in the *ydj1-151^{ts}* mutant mirrors the behavior of this substrate in the *ssa1-45^{ts}* mutant. Under permissive conditions a significant fraction of ΔssCG* is soluble while at restrictive conditions a major part of the protein is found in the pellet fraction (Fig. 20A and B). We have shown that ΔssCG* is nearly completely degraded in *SSA1* cells at 37℃ (Fig. 13A) despite the fact that under these conditions ΔssCG* partly precipitates (Fig. 20A). This indicates that Ssa1p may have the capacity to re-solubilize the precipitated material under the conditions tested. We looked for re-solubilization of ΔssCG* in *SSA1* and *ssa1-45^{ts}* cells in a cycloheximide decay experiment at 37℃ (Fig. 20D). As shown, within 30 min of cycloheximide treatment the amount of ΔssCG* material increases in *SSA1* cells but thereafter nearly completely disappears in the total fraction and in the pellet within 90min. In *ssa1-45^{ts}* cells the precipitated material persists under restrictive conditions. GFP carrying the mutated version of the proteasomal targeting sequence (GFP-cODC-C441A) is not eliminated by the proteasome (Hoyt *et al*, 2003). Indeed, the GFP-cODC-C441A protein accumulates in *SSA1* cells (Fig. 20E). However, in contrast to ΔssCG* (Fig. 20C), the accumulated material does not show any sign of aggregation.

3.2.9 Ubiquitination of misfolded proteins in the cytosol does not depend on Ssa1p and Ydj1p in yeast

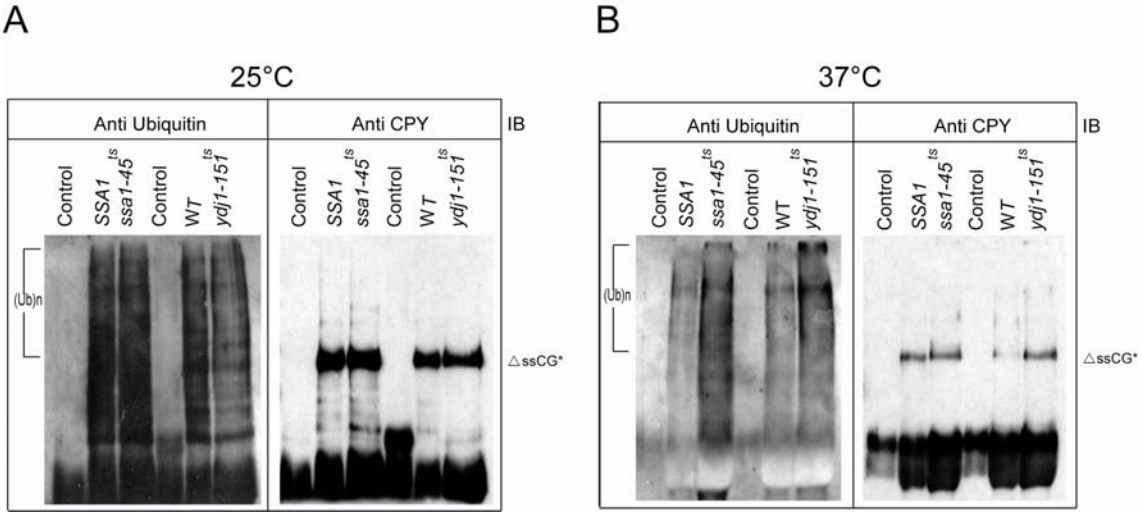

Figure 21. **The state of ubiquitinated misfolded proteins in wild type, *SSA1*, *ssa1-45^{ts}* and *ydj1-151^{ts}* cells at 25℃ and 37℃.** Cells harboring over-expressed ΔssCG* or an empty plasmid (control) were grown at 25℃ (A) and shifted to 37℃ (B) for 60 min prior to analysis. Cell extracts were immunoprecipitated with anti GFP antibody, separated by SDS-PAGE followed by immunoblotting and analyzed with anti ubiquitin or anti CPY antibodies.

With few exceptions, like ODC and the cyclin-dependent kinase inhibitor p21 (Sheaff *et al.*, 2000; Verma *et al.*, 2000; Liu *et al.*, 2003; Hoyt and Coffino, 2004), ubiquitination of substrates is required prior to their elimination via the proteasome (Heinemeyer *et al.*, 1991; Pickart, 2001; Wolf *et al.*, 2004). Several groups have shown that in mammalian cells a CHIP associated Hsp70 chaperone complex triggers ubiquitination of its protein clients and mediates proteasomal degradation (Connell *et*

al., 2001; Demand *et al.*, 2001; Jiang *et al.*, 2001; Murata *et al.*, 2001). We searched for ubiquitinated ΔssCG* material in mutant and wild type cells, under the experimental design of Figure 20, and analyzed the soluble fraction of the respective cell extracts. The buffer used for solubilization (sorbitol; Fig. 20 or Tris/HCl; Fig. 21) did not alter the experimental result (data not shown). While we find clearly similar amounts of ubiquitinated ΔssCG* in wild type and mutant cells at 25℃ (Fig. 21A), conditions which do not induce the mutant character, we see a considerably changed ubiquitin pattern of ΔssCG* at 37℃, which leads to the expression of the mutant phenotype of *ssa1-45*^{ts} and *ydj1-151*^{ts} cells. Interestingly, considerably more ubiquitinated ΔssCG* can be found in *ssa1-45*^{ts} and *ydj1-151*^{ts} under restrictive conditions compared to WT (*SSA1, SSA2, SSA3, SSA4*) and *SSA1* cells (Fig. 21B) despite the fact that the mutant cells show much less soluble ΔssCG* material (Fig. 20). This might indicate that ΔssCG* in the *SSA1* and wild type cells is completely degraded while degradation of the ubiquitinated material is retarded in the mutant cells.

3.2.10 The E2 proteins Ubc4p and Ubc5p are required for degradation of ΔssCG* but not the E3 ligases Doa10p and Der3p

We were also interested in the components of the ubiquitination machinery in the degradation pathway of cytosolic substrates. At present there are 13 ubiquitin conjugating enzymes known to exist in yeast. As seen Figure 22A, deletion of the ubiquitin conjugating enzymes Ubc4p and Ubc5p leads to a considerable stabilization of ΔssCG*, indicating involvement of Ubc4p and Ubc5p in the degradation of this misfolded cytoplasmic protein. As degradation is not completely halted in the

ubc4/ubc5 double deletion mutant, an overlapping E2 activity must be present for ubiquitination of ΔssCG*. In mammalian cells, CHIP has been discovered as an important E3 ligase involved in degradation of proteins in the cytoplasm (Connell *et al.*, 2001; Demand *et al.*, 2001; King *et al.*, 2001; Murata *et al.*, 2001; Cyr *et al.*, 2002; Esser *et al.*, 2004). In yeast cells no CHIP orthologue has been found yet. However there are a number of E3 ligases present in yeast cells. Besides its involvement in degradation of several ERQD substrates, the ER membrane located, E3 ligase Doa10p is required for degradation of Deg1-GFP, a cytoplasmic and nuclear substrate (Swanson *et al.*, 2001; Huyer *et al.*, 2004; Ravid *et al.*, 2006). However, degradation of ΔssCG* is independent of the function of the E3 ligase Doa10p (Fig. 22B). Degradation of ΔssCG* did also not require the second ER membrane located E3 ligase Der3/Hrd1p (Fig. 22B).

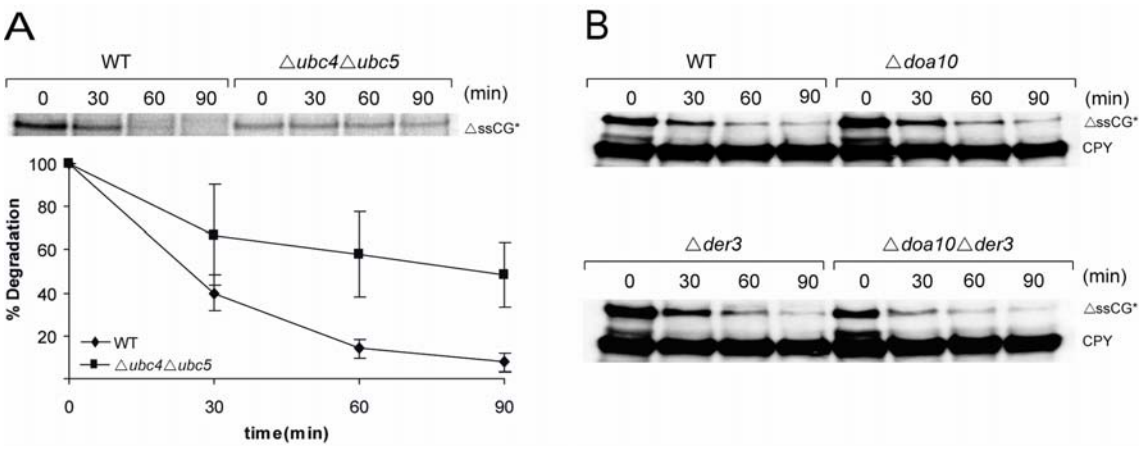

Figure 22. **Degradation of ΔssCG* requires the E2 proteins Ubc4p and Ubc5p but not the E3 ligases Doa10p and Der3p.** Pulse chase analysis was done in Δ*ubc4*Δ*ubc5* mutant cells (A) and cycloheximide decay experiments were performed in *Δdoa10Δder3* cells (B). CPY served as a loading control

4. Discussion

4.1. ERQD (Endoplasmic reticulum quality control and degradation)

The current model for ERQD and especially the degradation part of the process, ERAD, has evolved over the past 20 years and is based on data accumulated using a wide range of substrates with different structural and functional characteristics studied in different eukaryotic systems. This has given us a broad knowledge about what ERAD is and how it functions in general. On the other hand, it is hard to achieve a clear assignment of components of the degradation machinery to topologically diverse domains of misfolded substrate proteins. To address this question, we used a set of modular substrates with topologically defined domains carrying the same degradation motif. The set consists of a soluble misfolded protein of the ER lumen (CPY*), CPY* linked to a transmembrane domain (CT*), and CPY* fused to a transmembrane domain followed by the green fluorescent protein (CTG*). In this study we have confirmed that CT* and CTG* span the ER membrane. The N-terminal CPY* moiety is located in the ER lumen, whereas the C-termini of both proteins are in the cytoplasm. Fluorescence of the cytosolic GFP domain of CTG* shows that it is biologically active and, therefore, correctly folded (Fig. 5C). As expected, the misfolded CPY* domain prevents secretion of the proteins out of the ER. As shown previously for soluble CPY* (Hiller et al., 1996; Bordallo et al., 1998; Plemper et al., 1999a; Friedländer et al., 2000; Jarosch et al., 2002), degradation of CTG* and CT* is also dependent on ubiquitination by the ubiquitin conjugating enzymes Ubc1p and Ubc7p, together with the ubiquitin protein-

ligase complex Der3/Hrd1-Hrd3 (Taxis, 2002; Taxis *et al.*, 2003). Further components of the degradation apparatus in the cytosol are the AAA-ATPase complex Cdc48-Ufd1-Npl4 and the 26S proteasome (Taxis, 2002, Fig. 6 and Fig 8). We have come to the conclusion that the components mentioned above constitute the basic machinery for ERAD of soluble and membrane proteins alike, which display misfolded domains in the ER lumen. Further studies are necessary to unravel whether other types of misfolded configurations in ER lumenal domains exist, which would recruit a different degradative machinery. The machinery used for degradation differs, in most cases, in the E3 used for polyubiquitination. Degradation of substrates with misfolding in the ER lumen, like CPY* (CT* and CTG*) and Pdr5*, depend on the E3 complex Der3/Hrd1-Hrd3 (Bordallo et al., 1998; Plemper et al., 1998; Plemper et al., 1999a). However, no substrate tested up till now has shown complete stabilization in Δ*der3/hrd1* cells, which implies the involvement of another E3 or of a different pathway in degradation. Degradation of a protein without a lumenal domain, Ubc6p, is completely independent of the ubiquitin-protein-ligase complex Der3/Hrd1-Hrd3 (Walter *et al.*, 2001), but has been shown to depend on the ubiquitin-protein-ligase Ssm4/Doa10p (Swanson et al., 2001). Soluble and membrane bound CPY* differ in their requirement for the ER membrane protein Der1p (Taxis, 2002) and the ER lumenal Hsp70 chaperone Kar2p (Taxis, 2002 and Fig. 9): these proteins are only necessary for degradation of soluble CPY* (Knop et al., 1996a; Plemper et al., 1997). Kar2p, together with Jem1p and Scj1p, was shown to solubilize CPY* in the ER lumen (Nishikawa *et al.*, 2001). Several lines of evidences indicate that Der1p (Taxis, 2002) and Kar2p (Fig. 9) are not involved in the recognition of misfolded protein domains in the ER lumen or in further unfolding of misfolded proteins prior to dislocation into the cytosol. They may function in localizing

soluble substrates to the lumenal face of the ER membrane and/or to the vicinity of the translocon and keep them in a dislocation competent state (Pilon *et al.*, 1997; Plemper *et al.*, 1997; Nishikawa *et al.*, 2001). The proteasome, the Cdc48-Ufd1-Npl4 complex together with the E2s Ubc1p and Ubc7p and the E3 complex Der3/Hrd1p-Hrd3p constitute the core components of the ERAD machinery responsible for removal of ERAD substrates having their misfolded domains within the ER. Degradation of glycosylated, misfolded proteins depends also on the lectin Htm1/Mnl1p and Yos9p, regardless of their topology (Jakob *et al.*, 2001; Buschhorn *et al.*, 2004).

Since Hsp70-Hsp40 chaperone complexes are thought to be involved in preventing protein aggregation rather than in protein unfolding (Hartl and Hayer-Hartl, 2002), we searched for chaperones with unfolding activity. Hsp100 chaperones are known to have such an activity (Glover and Lindquist, 1998; Weber-Ban et al., 1999). We found that a yeast Hsp100, Hsp104p, is involved in CTG* degradation (Fig. 10). It is known that Hsp104p forms a complex with the Hsp70 and Hsp40 chaperones to solubilize protein aggregates and allow subsequent refolding (Glover and Lindquist, 1998). Our data show that only CTG*, a misfolded membrane protein with a tightly folded cytoplasmic GFP domain, requires the unfolding activity of the Hsp70-Hsp40-Hsp104 protein complex for proper degradation (Fig. 10 and Fig. 11). Neither soluble CPY* nor CT*, lacking a cytosolic domain, require this chaperone complex for their degradation (Taxis, 2002). During cycloheximide treatment the tightly folded GFP domain of CTG* lost the fluorescence signal in *SSA1* cells where it was degraded by the ubiquitin-proteasome system but the fluorescence of CTG* in *ssa1-45*^{ts} cells persist under these conditions (Fig 11A). Indeed polyubiquitinated CTG* material accumulated in the ER membrane fraction of *ssa1-45*^{ts} cells (Fig. 11B). These data imply that the ATPases of the

proteasomal 19S cap may not be enough to support unfolding of the tightly folded GFP domain of CTG*. Instead the chaperone activity of Ssa1p may be required for the dislocation of polyubiquitinated CTG* prior to the action of the Cdc48p-Ufd1p-Npl4p complex. This may also explain the longer half life of CTG* compared to that of CT* and CPY*. It is still not clear whether the chaperone activity of Ssa1p is limited to unfolding of the tightly folded GFP domain of CTG* or, whether it is also required for the dislocation of polyubiquitinated CTG* directly. It is possible that the tightly folded cytoplasmic GFP domain may disturb the access of the Cdc48p-Ufd1p-Npl4p complex to the polyubiquitinated CTG*.

We were further interested in knowing how a short-lived, cytosolic GFP protein behaves in these mutants. Using a Deg1-GFP_2 fusion construct (Lenk and Sommer, 2000), we assessed the involvement of Hsp70 chaperones in the breakdown of unstable GFP molecules. Interestingly, we found that, just like CTG*, proteasomal degradation of Deg1-GFP_2 also depends on the action of the Ssa1p (Fig. 12A). Additionally, we found that the chaperone activity is required for the proteasomal degradation of the regulatory protein fructose 1,6-bisphosphatase (FBPase) (Fig. 12B). The finding that the chaperone activity of Ssa1p is not limited to ERAD substrates indicates the general importance of this chaperone activity for the elimination of unwanted proteins by the ubiquitin-proteasome system in the cellular context.

4.2. CQD (Cytoplasmic quality control and degradation)

Misfolded proteins of the endoplasmic reticulum are eliminated by proteasomal degradation in the cytosol. After detection, retrotranslocation and ubiquitination at the cytosolic surface of the ER they are channelled to the proteasome via the trimeric AAA-ATPase complex Cdc48p-Ufd1p-Npl4p and the UBA-UBL domain proteins Dsk2p and Rad23p (Brodsky and McCracken, 1999a; Kostova *et al.*, 2003; Hirsch *et al.*, 2004; Medicherla *et al.*, 2004). It has been shown that degradation of a cytoplasmically localized derivative of CPY* devoid of the signal sequence required for ER import (△ssCPY*-GFP), did not depend on the Cdc48p-Ufd1p-Npl4p, Dsk2p and Rad23p pathway for proteasomal degradation (Medicherla *et al.*, 2004). It became, therefore, our aim to understand the mechanism of degradation of misfolded proteins in the cytoplasm.

We, therefore, sought to determine the components that are required for elimination of ΔssCG* in the cytoplasm. As shown in Figure 13, degradation of ΔssCG* requires the Hsp70 chaperone Ssa1p. Recent *in vitro* experiments have shown that the 26S proteasome is unable to unfold the strongly folded GFP moiety of several fusion proteins tested (Liu *et al.*, 2003). We constructed a signal sequence deleted, cytoplasmically localized ΔssCPY* molecule devoid of the GFP domain to inquire if unfolding of that domain is responsible for the Ssa1p requirement. Surprisingly, ΔssCPY* degradation also depended on Ssa1p function (Fig. 14C), indicating that this Hsp70 species has a more general function in the degradation of cytoplasmically located misfolded proteins. The finding that degradation of ΔssCPY* and ΔssCPY also require

Ssa1p points to the fact that the role of this chaperone is not limited to unfolding, but serves additional purposes. The degradation of GFP fused to the C-terminal 37 amino acids of ornithine decarboxylase (GFP-cODC) without the aid of Ssa1p implies that the proteasome has other means to unfold GFP (Fig. 15A and B). A C441A mutation in the C-terminal 37 amino acid tail of ODC abolishes degradation of the fusion protein GFP-cODC-C441A (Hoyt *et al.*, 2003). The 37 amino acid stretch of cODC, whether wild type or mutated is not recognized as a misfolded protein domain by the cell (Hoyt *et al.*, 2003) and therefore the fate of GFP-cODC is independent of Ssa1p. Interestingly, fusion of mutated ΔssCPY* to GFP-cODC (ΔssCG*-cODC) reimposes a dependence on the Ssa1 chaperone for degradation (Fig. 15C). Also, mutation of cODC does not lead to stabilization of ΔssCG*-cODC-C441A (Fig. 15D). Thus Ssa1p seems to function in the recognition of the misfolded ΔssCPY* domain of the fusion protein; its misfolded status dictates the route of elimination.

It has, recently, been shown that the *in vivo* efficiency of signal sequence-mediated protein segregation into the secretory pathway varies tremendously, ranging from >95% to < 60% in mammalian cells (Levine *et al.*, 2005). Remnant secretory proteins thus find themselves entrapped in the cytoplasm. As mislocalized proteins may be harmful to the cell, the fate of these proteins is of high interest. The usefulness of mutated CPY variants in defining degradation pathways impelled a test of the fate of wild type CPY remaining in the cytoplasm. Like ΔssCG* and ΔssCPY*, ER import incompetent wild type CPY is also rapidly degraded by the proteasome (Fig. 16A), indicating an altered structure that is recognized by the cytoplasmic proteolysis system. We reason that proper folding of the enzyme is most likely defective due to disturbed formation of disulphide bonds (Endrizzi *et al.*, 1994; Jamsa *et al.*, 1994) in the reducing environment

of the cytoplasm, as compared to the oxidative environment of the ER in which CPY normally assumes its native and active form. As shown for ΔssCG* (Medicherla *et al.*, 2004), glycosylation of the enzyme is also likely to be absent in the cytoplasm. Thus the cell is easily able to eliminate mislocalized secretory proteins, which cannot fold efficiently in the cytoplasmic environment, in this way avoiding their unwanted presence in the cytoplasm.

All three cytoplasmically localized CPY derivatives, whether mutated (ΔssCG*, ΔssCPY*) or wild type (ΔssCPY), required the Hsp70 chaperone Ssa1p for elimination. While our work was in progress McClellan *et al.* (2005a) reported the requirement of Ssa1p for degradation of misfolded von Hippel Lindau (VHL) tumor suppressor protein in the yeast cytoplasm. We therefore conclude that the need for Ssa1p is likely to be a general feature of degradation of misfolded proteins in the cytoplasm. A crucial role for Hsp70 function in the degradation of different substrates has also been shown in mammalian cells (for review see Esser *et al.* 2004). It is interesting that the molecular chaperone Hsc70 is normally ubiquitinated by the CHIP E3 ligase in mammalian cells (Demand *et al.*, 2001; Jiang *et al.*, 2001; Murata *et al.*, 2001). Modification of Hsc70 with ubiquitin chains may alter the functional properties of this chaperone. Therefore, we tested whether mutations of ubiquitin modification sites in *SSA1* had an influence on the chaperone activity in the degradation of △ssCG*. However, no alteration of degradation of ΔssCG* is seen in three mutant *SSA1* alleles, $SSA1^{K521R}$, $SSA1^{K536R}$ *and* $SSA1^{K521R/K536}$ tested (Fig. 17). The ubiquitin modification of Hsp70 may function as a targeting sequence or alter cellular signaling events rather than degradative process. The functional requirement for Ssa1p for substrate recognition does not seem to be limited to ubiquitin dependent substrates. It has been reported that overexpression of the

molecular chaperones Hsp70 and Hsp40 facilitate degradation of α-synuclein which is natively disordered and degraded by the proteasome in the absence of ubiquitin modification (Tofaris *et al.*, 2001; Muchowski *et al.*, 2005).

In contrast to degradation of the ERQD substrate CTG* which is dependent on the Hsp40 co-chaperones Hdj1p, Cwc23p and Jid1p but not Ydj1p (Taxis *et al.*, 2003), along with Ssa1p, elimination of the CQD substrate ΔssCG* depends on the co-chaperone Ydj1p (Fig. 18B) and is independent of the other three co-chaperones (Fig. 18A). Degradation of ΔssCPY* and ΔssCPY, too, is dependent on Ydj1p (Fig 18C and D). In their work on the degradation of misfolded von Hippel Lindau (VHL) tumor suppressor protein in the yeast cytoplasm, McClellan *et al.* (2005) reported that the Hsp70 co-chaperone Sti1/HOP is required for degradation of VHL. They also reported the necessity of the Hsp90 chaperone system for elimination of misfolded VHL. In addition, the participation of the Hsp110 chaperone Sse1p was found for degradation of misfolded VHL. Ydj1p was not required for elimination of misfolded VHL (McClellan *et al.*, 2005a). Surprisingly, except for Ssa1p, the requirement of factors required for elimination of the three cytosolic substrates tested in our work differs completely from the factors reported by McClellan *et al.* (2005) for degradation of VHL. Neither the Hsp90 family of chaperones nor the Hsp110 chaperone Sse1p is required for the degradation of ΔssCG* (Fig. 19B and D, Bolender, 2005). While the co-chaperone Sti1p/HOP is necessary for degradation of misfolded VHL (McClellan *et al.*, 2005a), this factor is not involved in ΔssCG* degradation (Fig. 19C, Bolender, 2005). In contrast, the Hsp40 co-chaperone Ydj1p is an important factor in ΔssCG* as well as ΔssCPY* and ΔssCPY elimination (Fig. 18 B-D). While McClellan *et al*. (2005) show

only a minor portion of insoluble misfolded VHL in cells devoid of the Hsp70 co-chaperone Sti1/HOP, the situation concerning ΔssCG* is again different.

In vitro analysis shows that in wild type cells harboring all four Hsp70 species of the Ssa type (Fig. 20B, WT) the majority of ΔssCG* is found in the soluble fraction when cells are grown either at 30℃ or 37℃. As expected, the *in vivo* fluorescence of ΔssCG* is distributed throughout the cytoplasm of these cells (Fig. 20C). In contrast, *in vitro* analysis at 30℃ of *SSA1* or *ssa1-45*^{ts} cells harboring only one functional Ssa-species shows that the insoluble portion of ΔssCG* increases, indicating that one Ssa-species is at its limits in keeping the misfolded protein soluble. At 37℃ the insolubility of ΔssCG* increases in *SSA1* cells and nearly all ΔssCG* material is insoluble in *ssa1-45* cells, which lack Ssa1p activity at this temperature (Fig. 20A). Similar results have been observed for ΔssCPY* and ΔssCPY (data not shown). This behaviour is reflected *in vivo* when analyzing the fluorescence of ΔssCG* (Fig. 20C). The fact that less aggregated ΔssCG* material is seen in the fluorescence images as compared to the solubility assay *in vitro* may be due to the presence of oligomeric ΔssCG* species *in vivo* which under *in vitro* conditions form insoluble precipitates. It is interesting to note that degradation of ΔssCG* is rapid and nearly complete in *SSA1* cells at 37℃ indicating that the precipitated material is susceptible to degradation (Fig. 13). It has been shown that the Hsp70 chaperone machinery is able to restructure and disaggregate protein aggregates *in vitro* (Zietkiewicz *et al.*, 2006). Here we show that Ssa1 is able to re-solubilize precipitated ΔssCG* material *in vivo* (Figure 20D). We also tested the involvement of Hsp104 and the small heat shock proteins Hsp26 and Hsp42 in the degradation process of ΔssCG*. Surprisingly none of them exhibited any effect (Figures 19E and F). Cells defective in the activity of the Hsp40 co-chaperone Ydj1p

also show increasing amounts of ΔssCG* aggregates (Fig. 20B and C). Degradation of ΔssCG* is not completely blocked in *ydj1-151*^{ts} cells at the non-permissive temperature of 37℃ (Figure 18B). The most likely explanation for this behaviour is that Ssa1p is active without Ydj1p and that this co-chaperone only augments the capacity of Hsp70 to disaggregate oligomeric and insoluble precipitates. The absence of Ydj1p dependency of misfolded VHL degradation may be due to the fact that this protein remains soluble in the cytoplasm and does not form aggregates (McClellan *et al.*, 2005a). The Hsp40 co-chaperones have a conserved J-domain which is proposed to interact with Hsp70 and have been shown to exhibit a protective function in experimental model protein aggregation (Schaffar *et al.*, 2004; Muchowski *et al.*, 2005; Novoselova *et al.*, 2005). This implies that Ydj1p cannot be only some "specificity factor" for protein recognition, but rather represents an Ssa1p linked activity enhancer. After substrate solubilization Ssa1p is able to perform the additional tasks of keeping the substrate soluble and delivering it to the proteasome. The discovery that the neuronal Hsc70 co-chaperone Hsj1p can act as a neuronal shuttling factor for sorting of chaperone clients to the proteasome supports this idea (Westhoff *et al.*, 2005).

When comparing the protein quality control process in the two major folding compartments of the cells, the cytoplasm and the ER, it is clear that similar mechanisms operate. As found for the Hsp70 class of Ssa-chaperones in the cytoplasm (Hartl *et al.*, 2002; Deuerling and Bukau, 2004), the major Hsp70 protein of the ER, BiP in mammalian cells (Sitia *et al.*, 2003) or Kar2p in yeast is required for protein folding (Simons *et al.*, 1995). When folding is not successful, Kar2p is necessary to prevent proteins from aggregating and to keep misfolded proteins of the ER in the soluble state (Nishikawa *et al.*, 2001), prior to their retrotranslocation into the cytoplasm and

degradation by the proteasome (Plemper *et al.*, 1997; Brodsky *et al.*, 1999b). These functions of Kar2p are also dependent on co-chaperones (Nishikawa *et al.*, 2001). As shown here and elsewhere (McClellan *et al.*, 2005a), Ssa1p together with its co-chaperones seems to have parallel functions in the cytoplasm.

Central components of CQD seem to be the Hsp70 chaperone Ssa1p (Fig. 13 and McClellan *et al*., 2005a), the ubiquitin conjugating enzymes Ubc4p and Ubc5p (Fig. 22A and McClellan *et al.,* 2005a) and the proteasome (Fig. 14 and McClellan *et al.,* 2005a). The ubiquitin protein ligase (E3) that functions in this system remains to be identified. We analyzed a subset of known ubiquitin protein ligases (E3's) Doa10p, Der3p (Fig. 22B), Rsp5p, Hul5p, Ufd4p and the SCF complex (data not shown). None of these ligases is involved in the degradation of the model substrate ΔssCG* in the cytoplasm. This suggests the involvement of a novel E3 in the degradation process of the misfolded proteins in the cytoplasm.

Our experiments show that the Hsp90 family of chaperones is not invariably needed for degradation of misfolded proteins (Figure 19B). In the case of misfolded VHL, Hsp90 action may be uniquely required to generate a specific conformation of this substrate, one that can subsequently be recognized by an ubiquitin ligase involved in quality control. The specific co-chaperone required for Ssa1p dependent ubiquitin-proteasome degradation of misfolded cytoplasmic proteins may depend on the function Ssa1p has to fulfil in this process. As only the soluble form of ΔssCG* can be degraded by the proteasome, we consider the polyubiquitinated ΔssCG* material in wild type and *SSA1* cells at 37℃ to be the steady state level of re-solubilized and not yet degraded ΔssCG* (Fig. 21B). As compared to wild type and Ssa1p cells, a considerably greater

amount of ubiquitinated soluble ΔssCG* material can be found in *ssa1-45^{ts}* and *ydj1-151^{ts}* cells under restrictive conditions (Fig. 21B) despite the fact that much less soluble ΔssCG* material is present in the mutant cells (Fig. 20A and B). From this one may conclude that ΔssCG* material ubiquitinated prior to the temperature shift to 37℃ may remain undegraded in the ubiquitinated state in the *ssa1-45^{ts}* or less well degraded in the *ydj1-151^{ts}* cells after the temperature shift, due to inactivation of the chaperone proteins. The fact that polyubiquitinated protein material accumulates in *ssa1-45^{ts}* mutant cells at the restrictive temperature of 37℃ despite the presence of an active proteasome (Fig. 21B) indicates that Ssa1p may have a function beyond solubilization of precipitated protein material or keeping misfolded proteins soluble. We conclude that Ssa1p is likely to have several functions. Ssa1p can unfold proteins (Taxis *et al.,* 2003 and Fig. 11), recognize misfolded protein domains (Fig. 15), solubilize (and keep soluble) aggregated misfolded proteins (Fig. 13A and Fig. 20D) and escort and deliver misfolded cytoplasmic proteins to the proteasome for degradation (Fig. 23). The finding of an interaction of Ssa1p with the 26S proteasome (Verma *et al.*, 2000, Coffino *et al.*, unpublished data) substantiates the validity of this last conclusion.

The cell must keep and maintain its homeostatic balance between folding intermediates and efficient elimination of terminally misfolded species for cell viability. Our understanding of protein quality control is not only important for scientific interest but also for therapies of a lot of human diseases. This study demonstrates the crucial role of the molecular chaperone machinery of the Ssa1 class on protein quality control in yeast cells. Further studies will be required for the identification of the E3/E4 ligases in this process and for revealing how molecular chaperones recruit different components for elimination of various types of misfolded proteins. In addition, the delivery

mechanism of misfolded proteins and the interaction mechanism between the 26S proteasome and the molecular chaperone complexed with the misfolded protein and probably other factors still remain to be discovered.

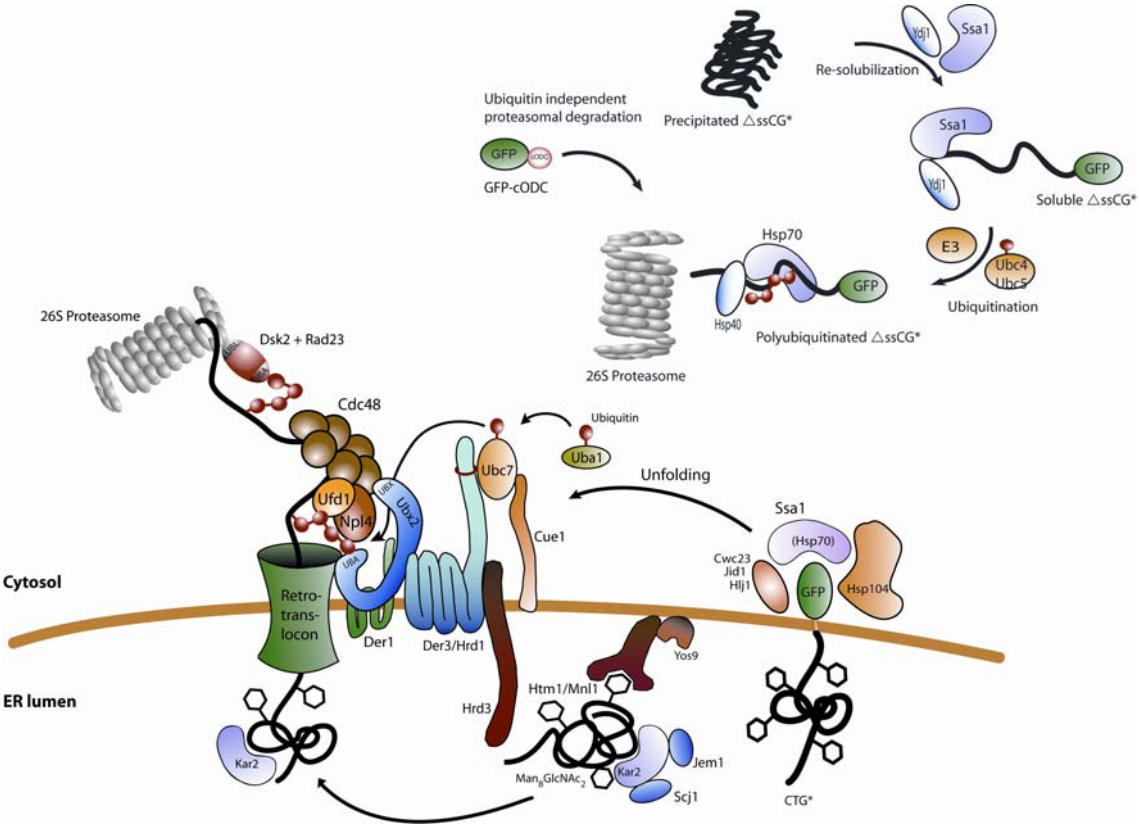

Figure 23. **Model of Protein Quality Control in yeast**. See text for details

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Curriculum vitae

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1999	B.S.c, Engineering in Food Science and Technology
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Academic Appointments

1999-2001	Research assistant in the Department of Microbiology
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2002-present	Teaching assistant for the Graduate level Biochemistry laboratory Course and for Diploma thesis works at the University of Stuttgart, Germany

Awards and Honors

1995-1999	University Grants Commission, scholarship in B.S.c, Daegu, Korea
2001	Winter Institute fellowship by the Japan International Science and Technology Exchange Center (JISTEC) in the National Institute of Bioscience and Human-Technology
2001	Summer Institute fellowship by the Deutscher Akademischer Austausch Dienst (DAAD) at the Institute of Biochemistry, University of Stuttgart

Research Expertise

Genetic and molecular biological manipulation of *S. cervisiae*

Molecular biological techniques

Expression of epitope tagged eukaryotic proteins in *E. coli* and *S. cervisiae*

Immunoprecepitation, SDS-PAGE and immunoblot analysis of proteins

Determining the ER localization of proteins

Detection of ubiquitinated proteins in various cellular compartments

Determination of the ER-associated degradation of proteins by pulse chase and cycloheximide decay analysis

Genome wide mutant screen in *S. cervisiae* by multi well transformation assay

Publication

Taxis C, Hitt R, **Park SH**, Deak PM, Kostova Z, and Wolf DH (2003). Use of Modular Substrates Demonstrates Mechanistic Diversity and Reveals Differences in Chaperone Requirement of ERAD. J Biol Chem. 278, 35903-35913

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Poster presentation

A genome-wide transcriptional analysis of *Saccharomyces cerevisiae* KNU5377 in response to various stresses. International meeting of Biochemistry and Molecular Biology 17-18 May, 2001 Seoul, Korea

Oxidative stress response of *Saccharomyce cerevisiae* KNU5377 to $H_20_{2..}$ 9th International Symposium on the Genetics of Industrial Microorganisms (GIM-2002), 1-5 July, 2002, Kyung-Ju, Korea

A Genomic screen identifies multiple new gene products involved in ER-associated Degradation (ERAD). Annual conference of the European Life Scientist Organization (ELSO), 20-23 September, 2003, Dresden, Germany

The cytoplasmic Hsp70 chaperone machinery subjects misfolded and ER import incompetent proteins to degradation via the ubiquitin-proteasome system. Annual Meeting of the American Society for Cell Biology, 10-15 December, 2005, San Francisco, USA

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Eidesstattliche Erklärung

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

Stuttgart, den 2. March 2007

Sae-Hun Park