The Cytoplasmic Hsp70 Chaperone Machinery Subjects Misfolded and Endoplasmic Reticulum Import-incompetent Proteins to Degradation via the Ubiquitin–Proteasome System

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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 degradation of two endoplasmic reticulum (ER)-import-defective mutated derivatives of carboxypeptidase yscY (Δ ssCPY* and Δ ssCPY*-GFP) and also examined the requirements for degradation of the corresponding wild-type enzyme made ER-import incompetent by removal of its signal sequence (Δ ssCPY). 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 Hsp70 cochaperone Ydj1p. Neither the Hsp90 chaperones nor Hsp104 or the small heat-shock proteins Hsp26 and Hsp42 are 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 Δ ssCPY* to GFP-cODC to form Δ ssCPY*-GFP-cODC reimposes 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 misfolded proteins in the ubiquitinated state to the proteasome for degradation.

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 multiprotein complexes. Translocation of proteins into the endoplasmic reticulum (ER) 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. This includes recognition of misfolding, prevention of protein aggregation, and facilitation of refolding of partially unfolded proteins due to stresses (Goldberg, 2003; Kleizen and Braakman, 2004). Terminally misfolded proteins have to be recognized and eliminated. This process is essential to all

cells. Misfolding leads to the exposure of hydrophobic patches in proteins, which may cause 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, Parkinson, or Creutzfeldt-Jakob disease in humans or bovine spongiform encephalopathy (BSE) in cattle (Kopito, 2000; Dobson, 2003; Goldberg, 2003; Barral *et al.*, 2004).

Selective protein degradation via the ubiquitin–proteasome system is a major pathway conserved throughout eukaryotic evolution (Hochstrasser, 1996; Varshavsky, 1997; Hershko and Ciechanover, 1998; Wolf and Hilt, 2004). Ubiquitination of proteins is mediated by three consecutive reactions: ubiquitin activation via an E1 enzyme, ubiquitin conjugation via E2 enzymes, and the action of ubiquitin protein ligases, E3's, which mediate the selection of substrate and initiate its ubiquitination. Quality control and degradation of secretory proteins (ERQD) as well as of cytoplasmic proteins is under intensive study (Plempner *et al.*, 1997; Sommer and Wolf, 1997; Brodsky and McCracken, 1999; Kostova and Wolf, 2003; Hirsch *et al.*, 2004; McClellan *et al.*, 2005b; Schafer and Wolf, 2005; Bukau *et al.*, 2006). Cytoplasmic degradation is pertinent not only to proteins native to the cytoplasm, but also to secretory proteins that fail to fold properly. Misfolded secretory proteins are recognized in the ER, prevented from continuing along the secretory pathway, retrotranslocated to the cytoplasmic side of the ER, polyubiquitinated, and delivered to the proteasome for degradation. This mechanism

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Table 1. Yeast strains used in this study

Name	Genotype	Source
YWO1	<i>Mataα ura3-52 leu2-3,2-112 his3 Δ200 lys2-801 trp1-1</i>	Seufert and Jentsch (1990)
YWO23	<i>Mataα ura3-52 leu2-3,2-112 his3 Δ200 lys2-801 trp1-1 Δubc4::HIS3 Δubc5::LEU2</i>	Seufert and Jentsch (1990)
YPH499Y	<i>Mata ura3-52 leu2-1 his3Δ200 trp1-63 lys2-801 ade2-101 prc1-1</i>	Hiller <i>et al.</i> (1996)
CMY762Y	<i>Mata cim3-1 ura3-52 leu2-1 his3 Δ200 prc1-1</i>	Hiller <i>et al.</i> (1996)
W303-1C	<i>Mataα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 prc1-1</i>	Knop <i>et al.</i> (1996)
YPK002	<i>W303-1C Δsnl1::KAN^R</i>	This study
YPD5	<i>W303-1C Δydj1-2::HIS3 LEU2::ydj1-151</i>	Taxis <i>et al.</i> (2003)
YPD21	<i>Mataα his3-11, 15 leu2-3, 112 ura3-52 trp1-81 lys2 prc1-1 Δssa2::LEU2 Δssa3::TRP1 Δssa4::LYS2</i>	Taxis <i>et al.</i> (2003)
YPD22	<i>YPD21 Δssa2::LEU2 Δssa3::TRP1 Δssa4::LYS2 ssa1-45</i>	Taxis <i>et al.</i> (2003)
YCT397	<i>Mata leu2-3,112 ura3-52 ade1-100 his4-519 prc1-1</i>	Jarosch <i>et al.</i> (2002)
YCT415	<i>YCT397 ufd1-1</i>	Jarosch <i>et al.</i> (2002)
W303-1B	<i>Mataα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100</i>	Chiang and Schekman (1991)
AGC14	<i>Mataα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 Δhsp26::LEU2 Δhsp42::Hyg^B</i>	Cashikar <i>et al.</i> (2005)
MHY501	<i>Mataα his3-200 leu2-3,112 ura3-52 lys2-801 trp1-1</i>	Swanson <i>et al.</i> (2001)
MHY1631	<i>MHY501 Δssm4/doa10::HIS3</i>	Swanson <i>et al.</i> (2001)
MHY1669	<i>MHY501 Δhrd1/der3::LEU2</i>	Swanson <i>et al.</i> (2001)
MHY1703	<i>MHY501 Δhrd1/der3::LEU2 Δssm4/doa10::HIS3</i>	Swanson <i>et al.</i> (2001)
YRH023	<i>W303-1C Δhsp104::KAN^R</i>	Taxis <i>et al.</i> (2003)
YRH030	<i>W303-1C Δsti1-1::HIS3</i>	Taxis <i>et al.</i> (2003)
YRH050	<i>W303-1C Δhsc82::KAN^R hsp82^{G170D}</i>	Taxis <i>et al.</i> (2003)
Y406-C	<i>Mataα ura3-52 leu2-3,112 his3-11,15 lys2 trp1-1 prc1-1</i>	Deak (1998)
Y420-C	<i>Y406-C Δssb1::LEU2 Δssb2::HIS3</i>	Deak (1998)
BY4743	<i>Mataα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0</i>	EUROSCARF
BY474 Δ sse1	<i>BY4743 Δsse1::kanMX4/Δsse1::kanMX4</i>	EUROSCARF

for delivering misfolded ER proteins to the proteasome makes use of a trimeric AAA-ATPase complex consisting of Cdc48p-Ufd1p-Npl4p and of two UBA-UBL-domain proteins, Dsk2p and Rad23p, which are able to dock to the proteasome (Hartmann-Petersen and Gordon, 2004; Elsasser and Finley, 2005). Such a mechanism ensures that misfolded soluble or membrane bound secretory proteins are not released into the cytoplasm, where aggregation would occur, but are escorted instead in a protein bound form to the proteasome for elimination (Medicherla *et al.*, 2004).

This broadly accepted view was in part inferred from experiments using the misfolded ER-luminal model substrate CPY* (Finger *et al.*, 1993; Hiller *et al.*, 1996; Schafer and Wolf, 2006). Studies of CPY* processing and degradation have been more recently extended to two of its membrane bound derivatives, CT* and CTG*, carrying the ER luminal CPY* module, a transmembrane domain (CT*), or, in addition, the green fluorescent protein GFP (CTG*) (Taxis *et al.*, 2003). During our studies on the delivery mechanism of these misfolded ER model substrates to the proteasome, we also studied the degradation requirements of the cytoplasmically located CPY* derivative Δ ssCPY*-GFP. This protein lacks a signal sequence directing it to the ER. Thus, in contrast to ER lumen misfolded proteins like CPY*, which makes a round trip from cytoplasm to ER and back, Δ ssCPY*-GFP is made and remains in the cytoplasm. Δ ssCPY*-GFP was also rapidly degraded via the proteasome but did not require the Cdc48p-Ufd1p-Npl4p AAA-ATPase complex nor 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 secretory protein. Recently it has been found in mammalian cells that the efficiency of protein compartmentalization into the secretory pathway is far from perfect. Because of 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. It was the aim of this study to unravel the agents that recognize misfolded cytoplasmically located proteins and deliver them to the proteasome.

MATERIALS AND METHODS

Yeast Strains and Plasmids

Yeast strains used in this study are summarized in Table 1. All other methods for yeast manipulation and genetic experiments were carried out using standard methods (Guthrie and Fink, 1991; Ausubel *et al.*, 1992). The *SNL1* gene in *W303-1C* was disrupted by PCR amplification of the *Δsnl1::KAN^R* fragment from strain BY4743 (EUROSCARF, Frankfurt, Germany) using the primer pairs SNL1 5' Primer (GACGAATATAAGGTCAAAGCTTCA) and SNL1 3' Primer (TTTATTTTGGTATGATTTTAGGCGA). Correct integration of the disrupted DNA was confirmed by PCR analysis and Southern blotting. The identity of DNA fragments generated by PCR was verified by sequencing. Detailed cloning strategies are available on request. The plasmid pRS316- Δ ssCPY*-GFP is described previously (Medicherla *et al.*, 2004). To generate the plasmid pZK116 expressing Δ ssCPY*, the signal sequence was removed from the CPY* allele in plasmid pRS316-CPY* (Kostova and Wolf, 2005) by a QuickChange-based (Stratagene, La Jolla, CA) PCR-mutagenesis approach. The DNA of cytoplasmically localized, N-terminally GFP fused CPY* (Δ ssGC*) was cloned in two steps. First, the *SphI* restriction site of the plasmid was introduced to the end of the CPY promoter in pZK116 by QuickChange (Stratagene)-based PCR mutagenesis, yielding pZK116m. Then the PCR-amplified 0.7-kb GFP DNA fragment prepared from plasmid pRS316- Δ ssCPY*-GFP as template was cloned into the *SphI* restriction site of pZK116m, generating plasmid pRS316- Δ ssGFP-CPY*. The PCR-amplified 0.75-kb DNA fragment of GFP_{uv} from p416-P_{ADH}-GFP_{uv} as template (Hoyt *et al.*, 2003) was cloned into pRS316- Δ ssCPY*-GFP between the *HpaI* and *EcoRI* restriction sites, yielding plasmid pRS316- Δ ssCPY*-GFP_{uv}. The PCR-amplified 1.2-kb partial fragment of PRC1 from pYEP13/PRC1, which encodes wild-type CPY was used as template and inserted into pZK116 between the *Bsu36I* and *EcoRI* restriction sites, generating pRS316- Δ ssCPY. The 3.4-kb ORF, which encodes Δ ssCPY*-GFP from plasmid pRS316- Δ ssCPY*-GFP was subcloned into the 2 μ plasmid pRS426 between the *Clal* and *EcoRI* restriction sites, leading to overexpression of Δ ssCPY*-GFP. The PCR-amplified 0.5-kb DNA fragment of GFP_{uv}-cODC or GFP_{uv}-cODC-C441A from p416P_{ADH}-GFP_{uv}425cODC or p416P_{ADH}-GFP_{uv}425cODC-C441A as templates (Hoyt *et al.*, 2003) were cloned

into respective sites of pRS316- Δ ssCPY*-GFP_{uv} between the MluI and EcoRI restriction sites, yielding pRS316- Δ ssCPY*-GFP_{uv}-cODC or pRS316- Δ ssCPY*-GFP_{uv}-cODC-C441A, respectively.

Antibodies

Polyclonal anti-rabbit CPY (Knop *et al.*, 1993), polyclonal anti-rabbit GFP antibodies (Molecular Probes, Eugene, OR) were used for immunoprecipitation of Δ ssCPY*-GFP* and its derivatives. Monoclonal anti-mouse CPY (Molecular Probes), polyclonal anti-rabbit GFP antibodies were diluted 1:10,000 for immunodetection. Monoclonal anti-mouse ubiquitin antibody (BabCO, Richmond, CA) was used at 1:2000 dilution for immunodetection.

Pulse-Chase Analysis

Pulse-chase experiments using cells expressing CPY* or CPY fusion proteins, respectively, cell breakage in buffer containing urea, and SDS were performed as described previously (Hiller *et al.*, 1996; Taxis *et al.*, 2003). Temperature-sensitive strains were grown at 25°C and shifted to restrictive temperature for labeling with 200 μ Ci of ³⁵S-Met at 37°C for 20 min. Cells were chased with excess of unlabeled chase media for the times indicated in the respective figure legends.

Cycloheximide Decay Experiments

Cells were grown in synthetic complete medium. Temperature-sensitive strains were shifted to restrictive temperature of 37°C for 60 min. Cycloheximide was added (0.5 mg/ml), and 2 OD₆₀₀ of cells were taken at the indicated time points. Cell extracts were prepared by alkaline lysis and subjected to SDS-PAGE followed by immunodetection (Hiller *et al.*, 1996; Taxis *et al.*, 2003).

Solubility Assay

Cells expressing Δ ssCG* were grown at 30°C and shifted to 37°C for 60 min before assay. Twenty OD₆₀₀ of yeast cells were harvested, washed once with four volumes of 20 mM sodium azide, and resuspended in 1 ml of ice-cold sorbitol lysis buffer (0.7 M sorbitol, 50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 1 μ g/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 precleared by centrifugation at 500 $\times$ g for 5 min at 4°C. Total protein (T) was precipitated from 400 μ l of lysate with TCA (11% final concentration). Total protein (T) was solubilized with 60 μ l of urea buffer (40 mM Tris-HCl, pH 6.8, 8 M urea, 5% SDS, 100 mM EDTA, pH 8, 200 μ g/ml bromophenol blue, 1.5% beta mercaptoethanol). In addition 400 μ l of lysate was spun in a Beckman T110 rotor (Fullerton, CA) at 130,000 $\times$ g for 30 min at 4°C. The supernatant was subjected to TCA precipitation and treated as soluble protein (S). The pellet of the 130,000 $\times$ g centrifugation step was washed once with sorbitol lysis buffer followed by solubilization with 60 μ l 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. Resolubilization of aggregated Δ ssCG* was tested as follows: After temperature shift of cells to 37°C for 1 h, cycloheximide was added to a final concentration of 0.5 mg/ml. Twenty OD₆₀₀ of cells were taken at the indicated time points, and the solubility assay was performed as stated above.

Fluorescence Microscopy

Cells overexpressing Δ ssCPY*-GFP or harboring an empty plasmid were grown at 30°C and shifted to 37°C for 60 min before viewing fluorescence in living cells. Cells were collected by centrifugation, washed once, and resuspended in fresh SC medium. The suspension, 2.2 μ l, was dropped onto a 76 $\times$ 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 $\times$ oil-immersion objective (Carl Zeiss, Thornwood, NY) and GFP filter.

Ubiquitination of Δ ssCG*

Fifty OD₆₀₀ of yeast cells overexpressing Δ ssCPY*-GFP or harboring an empty plasmid were grown at 25°C and shifted to 37°C for 60 min before analysis. Cells were washed once with ice-cold washing buffer (20 mM sodium azide, 2 mM PMSF, 20 mM NEM) and incubated for 10 min on ice. Cells were resuspended in ice-cold IP buffer (50 mM Tris-HCl, pH 7.5, 190 mM NaCl, 1.25% Triton X-100, 6 mM EDTA, 2 mM PMSF, 20 mM NEM), and 500 μ l of 0.5-mm glass beads were added. Cells were lysed by five pulses of 1-min duration in a Mini-bead beater, with cooling on ice between pulses. Lysates were cleared by centrifugation (130,000 $\times$ g, 30 min at 4°C), immunoprecipitated with anti-GFP, fractionated, and analyzed using anti-ubiquitin or anti-CPY.

RESULTS

Because degradation of the cytoplasmically localized substrate Δ ssCPY*-GFP(Δ ssCG*) by the proteasome did not re-

quire any of the cytoplasmic helper components of the ERAD pathway (Medicherla *et al.*, 2004), we searched for different chaperones that might be involved in its elimination. We reasoned that, as found for misfolded ER proteins, recognition, unfolding, escort, and delivery machineries must exist to deliver misfolded cytoplasmic proteins to the proteasome for degradation. 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 the requirement for Hsp70. We therefore assessed whether the Hsp70 chaperone machinery of the Ssa class had a crucial role in the degradation of Δ ssCPY*-GFP(Δ 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, 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 can be seen in Figure 1A, degradation of Δ ssCG* progresses with a half-life of 20–30 min in *SSA1* cells. Degradation of Δ ssCG* is nearly completely abolished in *ssa1-45^{ts}* cells under restrictive conditions. A similar almost complete dependence on Ssa1 for Δ ssCG* degradation is observed using antibodies directed against either CPY or GFP for immunoprecipitation (Figure 1A). 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. To test whether the position of the strongly folded GFP domain within Δ ssCG* had any effect on the degradation pattern and whether its context influenced the Ssa1p-dependence of degradation, we constructed Δ ssGFP-CPY* (Δ ssGC*), carrying GFP N-terminally fused to signal sequence deleted CPY*. As can be seen in Figure 1B, Δ ssGC* is degraded nearly as rapidly as Δ ssCG*, and lack of an active Ssa apparatus blocks degradation of this substrate as well. Also, fusion of a variant of GFP_{uv} that fluoresces more brightly than wild-type GFP at the C-terminus of Δ ssCPY* does not affect the half life of Δ ssCG* degradation (Figure 1C).

In vitro studies had shown that the 26S proteasome is unable to degrade the GFP moiety of certain fusion proteins, because of its strongly folded structure (Liu *et al.*, 2003). It was therefore possible that Ssa1p was only required for unfolding of the GFP moiety of Δ ssCPY*-GFP(Δ ssCG*) to allow its degradation by the proteasome *in vivo*. We constructed a CPY* protein without signal sequence, Δ ssCPY*. It is an ER import-incompetent CPY* species that due to mutation (G255R) is misfolded. As previously published for Δ ssCPY*-GFP (Medicherla *et al.*, 2004), the signal sequence deletion causes Δ ssCPY* to be located in the cytosol (data not shown). This protein is rapidly degraded by the proteasome: elimination of Δ ssCPY* is severely disrupted in the proteasome mutant *cim3-1* (Figure 2A). We have previously shown (Medicherla *et al.*, 2004) that the elimination of cytosolic Δ ssCPY*-GFP does not require the trimeric Cdc48p-Ufd1p-Npl4p complex. Testing the requirement of this trimeric complex for degradation in *ufd1-1* mutant shows that Cdc48p-Ufd1p-Npl4p is also not involved in the proteasomal elimination process of Δ ssCPY* (Figure 2B). As can be seen in Figure 2C, Δ ssCPY* is rapidly degraded in *SSA1* but not in *ssa1-45^{ts}* mutant cells under restrictive conditions. These experiments indicate that the Ssa machinery is needed for the degradation of misfolded proteins of the cytoplasm.

For elucidation if proteasomal degradation of the strongly folded GFP domain is indeed independent of Ssa helper proteins we tested the degradation of GFP linked to the

Figure 1. The Hsp70 chaperone machinery of Ssa1p is required for the degradation of cytoplasmically localized misfolded proteins. Pulse-chase analysis was done in *SSA1* and *ssa1-45^{ts}* cells. Cells expressing the substrates were lysed at the indicated times, and proteins were immunoprecipitated with anti CPY (A–C) or anti GFP (A), separated by SDS-PAGE, and analyzed using a PhosphorImager and ImagerQuaNT (Amersham Bioscience). Plotted data represent the mean values of three independent experiments. Substrates: A: ΔssCG*; B: ΔssGC*; C: ΔssCG*_{uv}. The ERQD substrate CPY* served as a control.

C-terminal 37 amino acids of mouse ornithine decarboxylase (cODC). This 37 amino acid C-terminal sequence has been shown to be a ubiquitin-independent transferable element, one with the capacity to direct diverse proteins to proteasomal degradation (Hoyt *et al.*, 2003; Zhang *et al.*, 2003, 2004). We tested the Ssa1 dependency of degradation of the fusion proteins GFP_{uv}-cODC and GFP-cODC. 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 under permissive or restrictive conditions of incubation (Figure 3, A 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) is again dependent on Ssa1p, as is ΔssCG* (Figure 3C). 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 Cys₄₄₁ to Ala₄₄₁ in this sequence causes a significant stabilization of ODC or of proteins to which cODC is attached (Hoyt *et al.*, 2003). However, the Cys₄₄₁ to Ala₄₄₁ mutation in ΔssCG*-cODC-C441A did not lead to stabilization but directed this protein to a form of degradation that relied on the Ssa1 protein (Figure 3D). Obviously, Ssa1p-directed degradation of the ΔssCPY* moiety of the protein dominates over the Ssa1p-independent cODC-directed degradation in the fusion protein.

It has been shown that the import of secretory proteins into the ER can be faulty (Levine *et al.*, 2005). Because the intracellular mislocalization of proteins may lead to severe defects, we were also interested in the question of how wild-type secretory proteins that 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. Using a multicopy plasmid for expression Blachly-Dyson and Stevens (1987) found ~90% of signal sequence deleted CPY in the cytosol and ~10% in the ER. We constructed a signal sequence deleted CPY (ΔssCPY) and ex-

pressed it from a single-copy plasmid. We found ΔssCPY, like ΔssCPY*, to be solely located in the cytosol (data not shown). The fact that in contrast to Blachly-Dyson and Stevens (1987) we did not find a small portion of ΔssCPY in the ER may be due to the different expression conditions. We analyzed the fate of ΔssCPY. The mislocalized and presumably misfolded ΔssCPY is rapidly degraded; its turnover is performed by the proteasome, as evidenced by the stabilization conferred by the proteasomal *cim3-1* mutant (Figure 4A). As is true for the mutated CPY species, degradation of ΔssCPY is independent of the trimeric Cdc48p-Ufd1p-Npl4p complex required for elimination of misfolded ER proteins (Figure 4B). However, elimination of ΔssCPY does require an intact Ssa1 protein (Figure 4C). 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. In the reducing environment of the cytoplasm, folding of CPY is likely to be defective due to disturbed formation of disulphide bonds (Endrizzi *et al.*, 1994; Jamsa *et al.*, 1994).

Hsp70 chaperones function in a complex with cochaperones of the Hsp40 family, which modulate the substrate specificity of the Hsp70s (Cheetham and Caplan, 1998; Johnson and Craig, 2001; Rudiger *et al.*, 2001; Fan *et al.*, 2003). In a previous study, we had shown that for degradation of the ERQD substrate CTG* the help of the Hsp40 cochaperones Hdj1p, Cwc23p, and Jid1p is required (Taxis *et al.*, 2003). However, for the degradation of cytoplasmic ΔssCG*, none of these Hsp40 cochaperones are needed (data not shown). In contrast, the Hsp70 cochaperone 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 in *ydj1-151^{ts}* mutant cells under restrictive conditions (Figure 5, A–C). Ydj1p is not required for any of the ERQD substrates derived from CPY* (Taxis *et al.*, 2003). It can be concluded that the CQD (cytoplasmic quality control and degradation) substrates ΔssCG*, ΔssCPY*, ΔssCPY,

Figure 2. 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 Δ ssCPY*. Cycloheximide was added ($t = 0$ min), and samples were collected at the indicated time points and subjected to SDS-PAGE, followed by immunoblotting. 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 Figure 1. The ERQD substrate CPY* served as a control.

and the ERQD substrate CTG* have different cochaperone requirements.

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, 2001). We tested a strain defective in this chaperone family (Δ ssb1 Δ ssb2) and found that they are dispensable for degradation of Δ ssCG* (Figure 6A). We also tested whether components of the Hsp90 chaperones were involved in degradation of Δ ssCG*. The yeast Hsp90 chaperone family consists of two proteins, Hsc82p and Hsp82p. They are associated with the cochaperone 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 Δ ssCG* (Figure 6B). Consequently, the Hsp70/Hsp90 cochaperone Sti1p/HOP has no effect on the degradation of Δ ssCG* (Figure 6C). It has been suggested that another major cytoplasmic chaperone, Hsp104, works together with the Hsp70s of the Ssa family and binds in an ATP-dependent manner to the Ssa1p-Ydj1p complex to unfold proteins (Parsell and Lindquist, 1993; Parsell *et al.*, 1994; Glover and Lindquist, 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 Δ ssCG* (Figure 6E). We were further interested in the involvement of the Hsp110 chaperone Sse1p in elimination of Δ ssCG*. The protein is a component of the Hsp90 chaperone complex and mediates degradation of misfolded VHL (McClellan *et al.*, 2005a). No function of Sse1p in Δ ssCG* degradation can be observed (Figure 6D). Two small heat-shock proteins, Hsp26 and Hsp42 are ubiquitous molecular chaperones that protect yeast cells from a variety of cellular stresses. In vitro they have been found to bind to unfolded proteins to form large cocomplexes and by this prevent their aggregation (Haslbeck *et al.*, 1999, 2004; Cashikar *et al.*, 2005). We tested the involvement of Hsp26 and Hsp42 in degradation of Δ ssCG*. As can be seen Figure 6F, degradation of Δ ssCG* was not affected by the absence of Hsp26 and Hsp42. Recently, BAG domain proteins were shown to interact with Hsp70 chaperones as a nucleotide exchange factor in the cytosol of higher eukaryotic cells. In mammalian cells, together with the E3 ligase CHIP, they are known to be partners in a degradative Hsp70 complex (Esser *et al.*, 2004). There exists a BAG-1 homologue in yeast, Snl1p, which functionally interacts with Hsp70 chaperones (Sondermann *et al.*, 2001, 2002). However, no alteration of degradation of Δ ssCG* is seen in *SNL1* deletion mutant cells (Figure 6G).

We tested whether the Ssa machinery has any function in keeping misfolded Δ ssCG* in the soluble state in the cyto-

Figure 3. Ubiquitin-independent degradation of GFP-cODC does not require Ssa1p activity, but its fusion to Δ ssCPY* 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), Δ ssCG*-cODC (C), and Δ ssCG*-cODC-C441A (D).

Figure 4. The fate 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 in the legend to Figure 2.

plasm. When testing wild-type cells harboring all four Ssa chaperones (Ssa1p, Ssa2p, Ssa3p, Ssa4p), most of the ΔssCG* protein is found in the soluble state, and this does not change when cells are shifted from 30 to 37°C (Figure 7B). As can be seen in Figure 7A, when *SSA1* cells containing solely Ssa1p are transferred from 30 to 37°C, the ΔssCG* material in the pellet increases, indicating aggregation of the misfolded protein material with increased temperature. The amount of precipitated ΔssCG* in *SSA1* cells varied somewhat in different experiments (data not shown). Apparently, in the absence of Ssa2, Ssa3, and Ssa4 the single Ssa1 species is functioning at or beyond its limits in keeping misfolded protein soluble under heat stress. However, analyzing the amount of soluble and precipitated cellular protein material in vitro may not be fully informative of the solubility properties of ΔssCG*, because in vitro conditions (buffer, salt, protein concentration, etc.) are very different from the cellular environment. We therefore analyzed the solubility of ΔssCG* in the different strains by fluorescence microscopy, thus visualizing the distribution pattern of the GFP moiety of the protein. As can be seen in Figure 6C, no precipitated ΔssCG* material can be seen at 37°C in wild-type cells containing all four Ssa species, regardless of whether ΔssCG* was expressed from a single-copy (data not shown) or multicopy plasmid (Figure 7C). In contrast, at 37°C some punctuated fluorescent dots, indicating 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°C (Figures 1, A–C). A dramatic increase in such precipitated fluorescent material appears under the restrictive conditions of 37°C in the *ssa1-45* and *ydj1-151* mutant cells. Under the restrictive con-

Figure 5. The Hsp70 cochaperone Ydj1p promotes the degradation of cytoplasmically localized misfolded proteins. Pulse-chase analysis was performed in wild-type (WT) and *ydj1-151^{ts}* cells expressing ΔssCG* (A), ΔssCPY* (B), and ΔssCPY (C).

ditions of 37°C in *ssa1-45^{ts}* mutant cells, we see most of the misfolded ΔssCG* material in the pellet (Figure 7A), and degradation is completely blocked (Figures 1, A 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, whereas at restrictive conditions a major part of the protein is found in the pellet fraction precipitated in cells (Figures 7, A and B). We have shown that ΔssCG* is nearly completely degraded in *SSA1* cells at 37°C (Figure 1A) despite the fact that under these conditions ΔssCG* partly precipitates (Figure 7A). This indicates that Ssa1p may have the capacity to resolubilize the precipitated material for degradation under the conditions tested. We tested resolubilization of ΔssCG* in *SSA1* and *ssa1-45^{ts}* cells in a cycloheximide decay experiment at 37°C (Figure 7D). As can be

Figure 6. 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 $\Delta ssCG^*$. Pulse-chase analysis was done in $\Delta ssb1\Delta ssb2$ (A), $\Delta hsc82hsp82^{G170D}$ (B), $\Delta hsp104$ (E), $\Delta hsp26\Delta hsp42$ (F), and $\Delta snl1$ (G) cells expressing $\Delta ssCG^*$, and cycloheximide decay experiments were performed in *sti1-1* (C) and $\Delta sse1$ (D) cells expressing $\Delta ssCG^*$ as described in the legend to Figure 2. PGK and CPY were served as a loading control.

seen, within 30 min of cycloheximide treatment the amount of $\Delta ssCG^*$ material increases in *SSA1* cells but thereafter nearly completely disappears in the total fraction and in the pellet within 90 min. In *ssa1-45^{ts}* cells the precipitated material persists, whereas GFP-cODC carrying the 37 amino acid targeting sequence of ODC for the proteasome is rapidly degraded by the enzyme (Hoyt *et al.*, 2003 and Figures 3A and 7E). 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 (Figure 7E). However, in contrast to $\Delta ssCG^*$ (Figure 7C) the accumulated material does not show any sign of aggregation.

With few exceptions, like ODC and the cyclin-dependent kinase inhibitor p21 (Sheaff *et al.*, 2000; Verma and Deshaies, 2000; Liu *et al.*, 2003; Hoyt and Coffino, 2004), ubiquitination of substrates is required before their elimination via the proteasome (Heinemeyer *et al.*, 1991; Pickart, 2001; Wolf and Hilt, 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 ubi-

quitinated $\Delta ssCG^*$ material in mutant and wild-type cells, under the experimental design of Figure 7, and analyzed the soluble fraction of the respective cell extracts. The buffer used for solubilization (Figure 7, sorbitol, or Figure 8, Tris/HCl) did not alter the experimental result (data not shown). Although we find clearly similar amounts of ubiquitinated $\Delta ssCG^*$ in wild-type and mutant cells at 25°C (Figure 8A), conditions that do not induce the mutant character, we see a considerably changed ubiquitin pattern of $\Delta ssCG^*$ material at 37°C, which leads to the expression of the mutant phenotype of *ssa1-45^{ts}* and *ydj1-151^{ts}* cells. Interestingly, considerably more ubiquitinated $\Delta ssCG^*$ can be found in *ssa1-45^{ts}* and *ydj1-151^{ts}* under restrictive conditions compared with WT (*SSA1*, *SSA2*, *SSA3*, *SSA4*) and *SSA1* cells (Figure 8B), despite the fact that the mutant cells show much less soluble $\Delta ssCG^*$ material (Figure 7). This might indicate that $\Delta ssCG^*$ in the *SSA1* and wild-type cells is completely degraded, whereas degradation of the ubiquitinated material is retarded in the mutant cells.

We were also interested in the components of the ubiquitination machinery in the degradation pathway. At present there are 13 ubiquitin-conjugating enzymes known to exist in yeast. As can be seen Figure 9A, deletion of the

Figure 7. Ssa1p and its cochaperone Ydj1p are required for rescue of aggregated ΔssCG*. Cells expressing ΔssCG* were grown at 30°C and shifted to 37°C for 60 min before 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 immunoblot. 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 overexpressed ΔssCG* or an empty plasmid were grown at 30°C and shifted to 37°C for 60 min before analysis. All the cells were visualized by fluorescence microscopy using equal exposure times and conditions. Resolubilization of aggregated ΔssCG* was assessed in SSA1 and *ssa1-45^{ts}* cells (D). After temperature shift of cells to 37°C for 1 h, cycloheximide was added to a final concentration of 0.5 mg/ml to block further protein synthesis. Twenty OD₆₀₀ of cells were taken at the indicated time points and treated as indicated for the above solubility assay. Immunoblot of 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°C as stated above (E).

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. Because 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 multitude of E3 ligases present in

Figure 8. The state of ubiquitinated misfolded proteins in wild-type, SSA1, *ssa1-45^{ts}*, and *ydj1-151^{ts}* cells at the different temperature of 25 and 37°C. The cells harboring overexpressed ΔssCG* or an empty plasmid (control) were grown at 25°C (A) and shifted to 37°C (B) for 60 min before 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.

Figure 9. Degradation of $\Delta ssCG^*$ requires the E2 proteins Ubc4p and Ubc5p but not the E3 ligases Doa10p and Der3p. Pulse-chase analysis was done in $\Delta ubc4 \Delta ubc5$ mutant cells (A) and cycloheximide decay experiments were performed in $\Delta doa10 \Delta der3$ cells (B) as described in the legend to Figure 2. CPY served as a loading control.

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 cytoplasmically and nuclear localized substrate (Swanson *et al.*, 2001; Huyer *et al.*, 2004; Ravid *et al.*, 2006). However, degradation of $\Delta ssCG^*$ is independent of the function of the E3 ligase Doa10p (Figure 9B). Degradation of $\Delta ssCG^*$ did also not require the second ER membrane-located E3 ligase Der3/Hrd1p (Figure 9B).

DISCUSSION

Misfolded proteins of the ER 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, 1999; Kostova and Wolf, 2003; Hirsch *et al.*, 2004; Medicherla *et al.*, 2004). Understanding of this mechanism, to a large extent, had been elaborated by using what turned out during time to be a model substrate for studying the ER-associated degradation process, mutated vacuolar carboxypeptidase yscY (CPY*) (Hiller *et al.*, 1996; Schafer and Wolf, 2006). We had shown that degradation of a cytoplasmically localized derivative of CPY* devoid of the signal sequence required for ER import ($\Delta ssCPY^*$ -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 $\Delta ssCG^*$ in the cytoplasm. As can

be seen in Figure 1, degradation of $\Delta ssCG^*$ requires the Hsp70 chaperone Ssa1p. Recent in vitro experiments had 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 $\Delta ssCPY^*$ molecule devoid of the GFP domain to inquire if unfolding of that domain is responsible for the Ssa1p requirement. Surprisingly, $\Delta ssCPY^*$ degradation also depended on Ssa1p function (Figure 2C), clearly indicating that this Hsp70 species has a more general function in the degradation of cytoplasmically located misfolded proteins. The present finding that degradation of $\Delta ssCPY^*$ and $\Delta ssCPY$ also require Ssa1p points to the fact that the role of this chaperone is not limited to unfolding, but serves additional purposes. 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 (Figure 3, A 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 $\Delta ssCPY^*$ to GFP-cODC ($\Delta ssCG^*$ -cODC) reimposes a dependence of the Ssa1 chaperone for degradation (Figure 3C). Also, mutation of cODC does not lead to stabilization of $\Delta ssCG^*$ -cODC-C441A (Figure 3D). Thus Ssa1p seems to function in the recognition of the misfolded $\Delta ssCPY^*$ 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. Because 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. As are $\Delta ssCG^*$ and $\Delta ssCPY^*$, ER import incompetent wild-type CPY is rapidly degraded by the proteasome (Figure 4A), indicating an altered structure that is recognized by the cytoplasmic proteolysis system. We reason that proper folding of the enzyme is most likely defective because of disturbed formation of disulphide bonds (Endrizzi *et al.*, 1994; Jamsa *et al.*, 1994) in the reducing environment of the cytoplasm, compared with the oxidative environment of the ER in which CPY normally assumes its native and active form. As shown for $\Delta ssCG^*$ (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 ($\Delta ssCG^*$, $\Delta ssCPY^*$) or wild-type ($\Delta ssCPY$), required the Hsp70 chaperone Ssa1p for elimination. While our work was in progress McClellan *et al.* (2005) 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). The functional requirement of Ssa1p for substrate recogni-

tion does not seem to be limited to ubiquitin-dependent substrates only. 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 and Wacker, 2005).

In contrast to degradation of the ERQD substrate CTG*, which, along with Ssa1p, is dependent on the Hsp40 cochaperones Hdj1p, Cwc23p, and Jid1p but not Ydj1p (Taxis *et al.*, 2003), elimination of the CQD substrate Δ ssCG* instead depends on the cochaperone Ydj1p and is independent of the other three cochaperones. Degradation of Δ ssCPY* and Δ ssCPY, too, is dependent on Ydj1p (Figure 5). In their work on the degradation of misfolded VHL tumor suppressor protein in the yeast cytoplasm, McClellan *et al.* (2005) reported the Hsp70 cochaperone Sti1/HOP to be 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 degradation of Δ ssCG* (Figure 6, B and D). Although the cochaperone Sti1/HOP is necessary for degradation of misfolded VHL (McClellan *et al.*, 2005a), this factor is not involved in Δ ssCG* degradation (Figure 6C). In contrast, the Hsp40 cochaperone Ydj1p is an important factor in Δ ssCG* as well as Δ ssCPY* and Δ ssCPY elimination (Figure 5). Although McClellan *et al.* (2005) show only a minor portion of insoluble misfolded VHL in cells devoid of the Hsp70 cochaperone Sti1/HOP, the situation concerning Δ ssCG* is again different.

In vitro analysis shows that in wild-type cells harboring all four Hsp70 species of the Ssa type (Figure 7B, WT) the majority of Δ ssCG* is found in the soluble fraction of cells grown either at 30 or 37°C. As expected, the fluorescence of Δ ssCG* is distributed throughout the cytoplasm of these cells in vivo (Figure 7C). In contrast, in vitro analysis at 30°C of SSA1 or *ssa1-45^{ts}* cells harboring only one functional Ssa-species shows that the insoluble portion of Δ ssCG* increases, indicating that one Ssa-species is at its limits in keeping the misfolded protein soluble. At 37°C the insolubility of Δ ssCG* increases in SSA1 cells, and nearly all Δ ssCG* material is insoluble in *ssa1-45* cells, which lack Ssa1p activity at this temperature (Figure 7A). Similar results have been observed for Δ ssCPY* and Δ ssCPY (data not shown). This behavior is reflected in vivo when analyzing the fluorescence of Δ ssCG* (Figure 7C). The fact that less aggregated Δ ssCG* material is seen in the fluorescence images compared with the solubility assay in vitro may be due to the presence of oligomeric Δ ssCG* species in vivo, which under in vitro conditions form insoluble precipitates. It is interesting to note that degradation of Δ ssCG* is rapid and nearly complete in SSA1 cells at 37°C, indicating that the precipitated material is susceptible to degradation (Figure 1). It has been shown that the Hsp70 chaperone machinery is able to remodel and disaggregate protein aggregates in vitro (Zietkiewicz *et al.*, 2006). Here we show that Ssa1 is able to resolubilize precipitated Δ ssCG* material in vivo (Figure 7D). We also tested the involvement of Hsp104 and the small heat-shock proteins Hsp26 and Hsp42 in the degradation process of Δ ssCG*. Surprisingly none of them exhibited any

effect (Figure 6, E and F). Cells defective in the activity of the Hsp40 cochaperone Ydj1p also show increasing amounts of Δ ssCG* aggregates (Figure 7, B and C). Degradation of Δ ssCG* is not completely blocked in *ydj1-151^{ts}* cells at the nonpermissive temperature of 37°C (Figure 5). The most likely explanation for this behavior is that Ssa1p is active without Ydj1p and that this cochaperone only augments the capacity of Hsp70 chaperone 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 cochaperones 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 and Wacker, 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 obviously able to perform the additional tasks of keeping the substrate soluble and delivering it to the proteasome. The discovery that the neuronal Hsc70 cochaperone 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 obvious that similar mechanisms operate. As found for the Hsp70 class of Ssa-chaperones in the cytoplasm (Hartl and Hayer-Hartl, 2002; Deuerling and Bukau, 2004), the major Hsp70 protein of the ER, BiP in mammalian cells (Sitia and Braakman, 2003) or Kar2p in yeast, is required for protein folding (Simons *et al.*, 1995). In case folding is not successful, Kar2p is necessary to prevent proteins from aggregation and keep soluble misfolded proteins of the ER in the soluble state (Nishikawa *et al.*, 2001), to finally allow their retrotranslocation into the cytoplasm and degradation by the proteasome (Plemper *et al.*, 1997; Brodsky *et al.*, 1999). These functions of Kar2p are also dependent on cochaperones (Nishikawa *et al.*, 2001). As shown here and elsewhere (McClellan *et al.*, 2005a), Ssa1p together with its cochaperones seems to have parallel functions in the cytoplasm.

Central agents of CQD seem to be the Hsp70 chaperone Ssa1p (Figures 1 and 2 and McClellan *et al.*, 2005), the ubiquitin-conjugating enzymes Ubc4p and Ubc5p (Figure 9A and McClellan *et al.*, 2005), and the proteasome (Figure 2 and McClellan *et al.*, 2005). 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 (Figure 9B), Rsp5p, Hul5p, Ufd4p, and the SCF complex (data not shown). None of these ligases is involved in the degradation of the model substrate Δ ssCG* in the cytoplasm. This suggests the involvement of a novel E3 in 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 6B). In the case of degradation 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 cochaperone required for Ssa1p-dependent ubiquitin-proteasome degradation of misfolded cytoplasmic proteins may depend on the function Ssa1p has to fulfill in this process. Because only the soluble form of Δ ssCG* can be degraded by the proteasome, we consider the

Figure 10. Model of protein quality control in the cytoplasm. See text for details.

polyubiquitinated Δ ssCG* material in wild-type and *SSA1* cells at 37°C to be the steady state level of resolubilized and not yet degraded Δ ssCG* (Figure 8B). Compared with wild-type and *Ssa1p*-proficient cells, a considerably greater amount of ubiquitinated soluble Δ ssCG* material can be found in *ssa1-45^{ts}* and *ydj1-151^{ts}* cells under these restrictive conditions (Figure 8B), despite the fact that much less soluble Δ ssCG* material is present in the mutant cells (Figures 7, A and B). From this one may conclude that Δ ssCG* material ubiquitinated before the temperature shift of cells to 37°C may remain undegraded in the ubiquitinated state in the *ssa1-45^{ts}* cells or less well degraded in the *ydj1-151^{ts}* mutant after the temperature shift, because of inactivation of the chaperone proteins. The fact that polyubiquitinated protein material accumulates in *ssa1-45^{ts}* mutant cells at the restrictive temperature of 37°C, despite the presence of an active proteasome (Figure 8B) 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), recognize misfolded protein domains (Figure 3), solubilize (and keep soluble) aggregated misfolded proteins (Figures 1A and 7D), and escort and deliver misfolded cytoplasmic proteins to the proteasome for degradation (Figure 10). The finding of an interaction of *Ssa1p* with the 26S proteasome (Verma *et al.*, 2000; Coffino, P., and Maxwell, R. A., unpublished data) substantiates the validity of this last conclusion.

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