

Distinct Machinery Is Required in *Saccharomyces cerevisiae* for the Endoplasmic Reticulum-associated Degradation of a Multispanning Membrane Protein and a Soluble Luminal Protein*

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The folding and assembly of proteins in the endoplasmic reticulum (ER) lumen and membrane are monitored by ER quality control. Misfolded or unassembled proteins are retained in the ER and, if they cannot fold or assemble correctly, ultimately undergo ER-associated degradation (ERAD) mediated by the ubiquitin-proteasome system. Whereas luminal and integral membrane ERAD substrates both require the proteasome for their degradation, the ER quality control machinery for these two classes of proteins likely differs because of their distinct topologies. Here we establish the requirements for the ERAD of Ste6p*, a multispanning membrane protein with a cytosolic mutation, and compare them with those for mutant form of carboxypeptidase Y (CPY*), a soluble luminal protein. We show that turnover of Ste6p* is dependent on the ubiquitin-protein isopeptide ligase Doa10p and is largely independent of the ubiquitin-protein isopeptide ligase Hrd1p/Der3p, whereas the opposite is true for CPY*. Furthermore, the cytosolic Hsp70 chaperone Ssa1p and the Hsp40 co-chaperones Ydj1p and Hlj1p are important in ERAD of Ste6p*, whereas the ER luminal chaperone Kar2p is dispensable, again opposite their roles in CPY* turnover. Finally, degradation of Ste6p*, unlike CPY*, does not appear to require the Sec61p translocon pore but, like CPY*, could depend on the Sec61p homologue Ssh1p. The ERAD pathways for Ste6p* and CPY* converge at a post-ubiquitination, pre-proteasome step, as both require the ATPase Cdc48p. Our results demonstrate that ERAD of Ste6p* employs distinct machinery from that of the soluble luminal substrate CPY* and that Ste6p* is a valuable model substrate to dissect the cellular machinery required for the ERAD of multispanning membrane proteins with a cytosolic mutation.

Protein production in mammalian cells is a remarkably imprecise process, with as many as 30% of proteins targeted for degradation during or shortly after synthesis (1). These degraded proteins likely include faulty proteins, which must be

removed to prevent detrimental effects to the cell. Similarly, the protein products of mutant genes must also be disposed of, as the accumulation of misfolded proteins has been associated with a number of human diseases including α 1-antitrypsin deficiency and Huntington's disease (reviewed in Refs. 2–5). Protein folding is monitored by cellular “quality control” systems throughout the cell (6, 7), and perhaps the best understood system is ER¹ quality control (ERQC), in which both soluble and membrane proteins destined for ER exit are inspected. Proteins that fail to fold or assemble correctly are retained in the ER and are ultimately degraded in a process known as ER-associated degradation (ERAD) that involves ubiquitination of ERAD substrates and degradation by the cytosolic proteasome (reviewed in Refs. 8–10).

Studies of the cellular machinery that mediates ERAD have been greatly facilitated by the use of model ERAD substrates. In yeast, an ER-retained mutant form of the soluble glycosylated vacuolar protease carboxypeptidase Y, CPY*, has been studied as a model luminal ER quality control substrate (11), whereas the hydroxymethylglutaryl-CoA reductase Hmg2p has been especially useful in dissecting the sterol-regulated degradation of a multispanning ER membrane protein (12). In mammalian cells, a mutant form of CFTR, a multispanning membrane protein, was one of the first ERAD substrates shown to undergo proteasome-mediated degradation (13, 14). Other model substrates include the major histocompatibility complex class I heavy chain in the presence of the human cytomegalovirus US11 protein (15), and the unassembled T cell antigen receptor α chain (16), both of which are type I membrane proteins. Studies of these and other model proteins have identified the ubiquitin-proteasome system, whose components reside in the cytosol or on the cytosolic face of the ER membrane, as the ultimate mediator of ERAD.

Whereas all ERAD substrates, whether luminal or membrane, are degraded by the proteasome, there are differences in how these classes of substrates are handled prior to degradation. For example, certain studies in yeast suggest that luminal ERAD substrates like CPY* may undergo ER to Golgi trafficking and are retrieved prior to degradation, whereas membrane proteins are retained in the ER (17, 18), although these conclusions are not universally accepted (19). It has been proposed recently that the degradative pathway for an ERAD substrate

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¹ The abbreviations used are: ER, endoplasmic reticulum; ERAD, ER-associated degradation; CPY*, mutant form of carboxypeptidase Y; ERQC, ER quality control; ERAC, ER-associated compartment; GFP, green fluorescent protein; E3, ubiquitin-protein isopeptide ligase; E2, ubiquitin-conjugating protein; HA, hemagglutinin; CFTR, cystic fibrosis transmembrane conductance regulator.

TABLE I
 Yeast strains used in this study

Strain	Relevant genotype	Ref./source
BWY46	<i>MATα ade2 his3 leu2 ura3 trp1 can1</i>	C. Stirling
BWY464	<i>MATα ade2 his3 leu2 ura3 trp1 can1 ssh1::TRP1</i>	C. Stirling
BY4741	<i>MATα his3 leu2 met15 ura3</i>	Open Biosystems, Huntsville, AL
MHY500	<i>MATα his3 leu2 ura3 lys2 trp1</i>	33
MHY552	<i>MATα his3 leu2 ura3 lys2 trp1 ubc6::HIS3 ubc7::LEU2</i>	33
MHY1685	<i>MATα his3 leu2 ura3 lys2 trp1 doa10Δ::HIS3</i>	37
MHY1702	<i>MATα his3 leu2 ura3 lys2 trp1 doa10Δ::HIS3 hrd1Δ::LEU2</i>	M. Hochstrasser
MHY2822	<i>MATα his3 leu2 ura3 lys2 trp1 hrd1Δ::LEU2</i>	M. Hochstrasser
MS10	<i>MATα ura3-52 leu2-3,112 ade2-101 KAR2</i>	76
MS193	<i>MATα ura3-52 leu2-3,112 ade2-101 kar2-133</i>	76
RSY156	<i>MATα leu2-3,112 ura3-52 pep4-3</i>	50
RSY533	<i>MATα leu2-3,112 ura3-52 pep4-3 ade2 sec61-2</i>	50
SM3385	<i>MATα his3 leu2 ura3 lys2 trp1 ste6Δ5 ubc6::HIS3</i>	This study
SM3387	<i>MATα his3 leu2 ura3 lys2 trp1 ste6Δ5 ubc7::LEU2</i>	This study
SM3388	<i>MATα his3 leu2 ura3 lys2 trp1 ste6Δ5 ubc6::HIS3 ubc7::LEU2</i>	This study
SM3390	<i>MATα his3 leu2 ura3 lys2 trp1 ste6Δ5</i>	This study
SM4177	<i>MATα his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2 ssa1-45 ssa2-1::LEU2 ssa3-1::TRP1 ssa4-2::LYS2</i>	45
SM4247	<i>MATα his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2 SSA1 ssa2-1::LEU2 ssa3-1::TRP1 ssa4-2::LYS2</i>	45
SM4783	<i>MATα ura3-52 leu2-3,112 ade2-1 trp-1 his3 cdc48-3</i>	T. Rapoport
SM4820	<i>MATα his3 leu2 met15 ura3 doa10Δ::kanMX</i>	Open Biosystems
SM4823	<i>MATα his3 leu2 met15 ura3 hrd1Δ::kanMX</i>	Open Biosystems
SM4824	<i>MATα his3 leu2 met15 ura3 hrd3Δ::kanMX</i>	Open Biosystems
SM4947	<i>MATα ade2 his3 leu2 ura3 trp1 can1-100</i>	T. Lithgow
SM4949	<i>MATα ade2 his3 leu2 ura3 trp1 ydj1-2::HIS3 ydj1-151::LEU2 hlj1::TRP1</i>	T. Lithgow
SM5129	<i>MATα his3 leu2 met15 ura3 ssh1Δ::kanMX</i>	Open Biosystems
W303a	<i>MATα ura3-52 leu2-3,112 ade2-1 trp-1 his3</i>	T. Rapoport
YJL183	<i>MATα ura3-Δ99 ade2-101 leu2Δ1 his3-200 trp1-Δ99</i>	D. Ng

is determined by whether the misfolded lesion is in the ER lumen or in the cytosol (20). Thus, a membrane protein with a luminal lesion would engage similar machinery as for a soluble luminal substrate, whereas a membrane protein with a cytosolic lesion would require distinct machinery. Many multispanning membrane proteins, including transporters, permeases, and channels, have large cytosolic domains and only small luminal/extracellular loops; thus, if such a protein is misfolded, there is a high probability that its lesion would be cytosolic as the bulk of the protein is in the cytosol. Consequently, the cellular machinery required for the ERAD of these membrane proteins would likely differ from that required for the ERAD of luminal substrates because the misfolded domains of these two classes of proteins are on opposite sides of the ER membrane.

To analyze further differences in ERAD of luminal and transmembrane proteins in yeast, we have taken advantage of a mutant form of Ste6p, an ATP-binding cassette transporter that normally functions at the plasma membrane to export the mating pheromone α -factor from cells. We previously identified mutant forms of Ste6p that result in a mating defect because of their ER retention and subsequent ERAD (21). Unexpectedly, prior to their being degraded, a subset of these mistrafficked Ste6p mutant proteins induce the formation of, and localize exclusively to, a quality control subcompartment of the ER called the ER-associated compartment (ERAC) (22), suggesting that there is a specific sorting and retention mechanism directing certain misfolded proteins out of the ER proper and into ERACs. The ERAC-localized mutant proteins are degraded at varying rates, with the least stable one ($t_{1/2} \sim 5$ –10 min) being Ste6-166p (21). This mutant protein, referred to here as Ste6p*, has a premature termination codon at position 1249 (Q1249X), resulting in deletion of the final 52 amino acids in the C-terminal cytosolic tail (Fig. 1A). The fast turnover of Ste6p*, coupled with its large cytosolic loops and the cytosolically located mutation, suggested to us that Ste6p* would make an ideal model protein to investigate how ERAD of this class of membrane proteins differs from that of luminal proteins. In fact, in a previous study we showed that degradation of Ste6p* is dependent on the ubiquitin-proteasome system (21), and we and others (17, 21) have shown that its degradation does not require ER to Golgi transport.

Here we compare the effects of mutations in specific components of the ERAD machinery on the turnover of Ste6p* versus the luminal misfolded substrate CPY*. We find that Ste6p* and CPY* employ overlapping but distinct ubiquitination machinery, with key differences in their E3 ubiquitin ligase requirement and chaperone dependence. Furthermore, ERAD of Ste6p* does not appear to be dependent on the Sec61p translocator for dislocation, in contrast to CPY*. However, the ERAD pathways for Ste6p* and CPY* converge after ubiquitination, but before proteasomal degradation, in that both substrates require the ATPase Cdc48p for their efficient degradation. Thus, the ERAD pathway for a multispanning membrane protein with a cytosolically located mutant domain (Ste6p*) has overlapping but distinct requirements from that of a mutant luminal protein (CPY*).

EXPERIMENTAL PROCEDURES

Strains, Media, and Growth Conditions—The yeast strains used in this study are listed in Table I. Plate and liquid complete, drop-out, or minimal media were prepared as described (23, 24). Yeast strains and cultures were grown at 30 °C, except for temperature-sensitive strains that were grown at room temperature (24 °C).

Strain and Plasmid Construction—Yeast transformations were performed by the method of Elble (25). Plasmid manipulations were performed in the *Escherichia coli* strains DH5 α (26) and MH1 (27) using standard media and techniques. The *ubc6 Δ* and *ubc7 Δ* strains were generated from MHY500 and MHY552. First, *STE6* was disrupted in the wild-type *MAT α* strain MHY500 by a one-step gene replacement, and the resulting strain (SM2948) was transformed with pSM192 (*CEN URA3 STE6*) (28) to permit mating with the *ubc6 Δ ubc7 Δ MAT α* strain MHY552. After selection for diploids, plasmid-free derivatives were identified by using 5-fluoroorotic acid and sporulated and dissected to recover the desired genotypes (*i.e.* *MAT α ste6 Δ* with either or both the *ubc6 Δ* and *ubc7 Δ* mutations).

The plasmids pSM1082 (2μ *URA3 ste6-166::HA*) and pSM1083 (*CEN URA3 ste6-166::HA*) have been described previously (21). The plasmid pSM1694 (2μ *URA3 ste6-166::HA::GFP*) was generated from pSM1081 (2μ *URA3 ste6-166::HA*) (21) by homologous recombination of the GFP open reading frame into the triple HA tag. The resulting plasmid contains the *STE6* promoter and *STE6* open reading frame, ending at the Q1249X termination codon, with GFP and two flanking HA epitope tags inserted in the first luminal loop of Ste6p* (between codons 68 and 69) (Fig. 1A). Plasmid pSM1912 (2μ *URA3 P_{PGK} ste6-166::HA::GFP*) was constructed by amplifying the *ste6-166* open reading frame (including the GFP and HA tags) from pSM1694 by PCR, introducing XmaI

and SacII sites at the 5' and 3' ends, respectively. The resulting PCR product was digested with XmaI and SacII and cloned into the same sites in pSM703 (29), which contains the *PGK* promoter. The constitutive *PGK* promoter permits expression of Ste6p* not only in *MATa* cells but also in *MAT α* cells in which the endogenous Ste6p promoter is repressed. The level of Ste6p* expression with the *PGK* promoter is equivalent to that with its endogenous promoter.² The plasmid pSM1911 (2 μ *URA3 P_{PGK} ste6-166::HA*) was constructed in an analogous manner to pSM1912, except that the *ste6-166* open reading frame was amplified from pSM1082, which contains the entire *STE6* sequence including the codons downstream of the Q1249X mutation, and has a triple HA tag (and no GFP) in the first luminal loop. The inserts in both pSM1911 and pSM1912 were sequenced to confirm that no mutations were introduced by PCR. The plasmid pSM1763 (*CEN URA3 CPY*::HA*) was a generous gift from Davis Ng (University of Pennsylvania) (30).

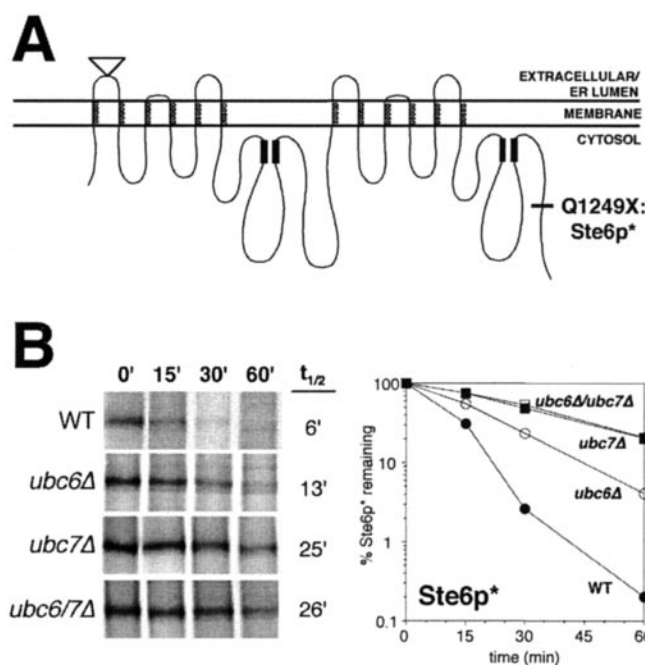
To create strains for examining the localization of Doa10p and Ubc7p, a C-terminal GFP tagging cassette was used (31). The plasmid pFA6a-GFP(S65T)-HIS3MX6 was used as a template for PCR to amplify a fragment containing the *GFP* ORF followed by the *Schizosaccharomyces pombe his5+* gene. The PCR product also contained 40-bp flanking sequences homologous to the 3' end of *DOA10* or *UBC7* to direct homologous recombination immediately after the final codon (and before the stop codon) of *DOA10* or *UBC7*. The PCR product was transformed into BY4741, and His⁺ transformants were selected. Correct integration was verified by PCR, using a forward primer near the 3' end of *DOA10* or *UBC7* and a reverse primer within the *GFP* sequence.

Metabolic Labeling Experiments—Cells were pulse-labeled and immunoprecipitated essentially as described (21). Briefly, log phase cells were pulse-labeled for 10 min with 30 μ Ci of ³⁵S-Express protein labeling mix (PerkinElmer Life Sciences) per 1 A₆₀₀ unit of cells; the label was chased with excess cold cysteine/methionine, and 2.5 A₆₀₀ units of cells were removed at the indicated times. Ste6p* and CPY* were immunoprecipitated with 12CA5 mouse anti-HA monoclonal antibodies (Roche Applied Science) bound to protein A-Sepharose beads (Amersham Biosciences) and resolved on SDS-PAGE (8 and 10%, respectively). Proteins were visualized and quantitated by using a PhosphorImager and Quantity One software (Bio-Rad), and half-lives were calculated from exponential curve fits using Kaleidagraph (Synergy Software, Reading, PA). All experiments were repeated at least three times with similar results; a representative experiment is shown in each case. No difference was observed when the turnover of Ste6p*-HA-GFP and Ste6p*-HA was compared in wild-type or ERAD mutant strains used in this study.²

Fluorescence Microscopy—Cells expressing Ste6p*-GFP were grown to mid-log phase and examined at $\times 100$ magnification on concanavalin-A-coated slides using a Zeiss Axioskop microscope equipped with fluorescence and Nomarski optics (Zeiss, Thornwood, NY). The excitation and emission filters were 470 nm (40 nm bandwidth) and 525 nm (50 nm bandwidth), respectively. Images were captured with a Cooke CCD camera and IP Lab Spectrum software (Biovision Technologies, Inc., Exton, PA).

RESULTS

ERAD of Ste6p* Depends on the E3 Doa10p—Protein ubiquitination requires the combined action of E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases (32). The E2 enzymes Ubc6p and Ubc7p have been shown to play a role in the degradation of almost all luminal and transmembrane ERAD substrates, with Ubc7p generally being the major E2, and the requirement for Ubc6p being more variable (10). We previously found that deletion of *UBC6* and *UBC7* together results in stabilization of Ste6p* (21). Here we examined the effect of single deletions of these E2 genes. We found that deletion of either *UBC6* alone or *UBC7* alone stabilized Ste6p* by approximately 2- and 4-fold, respectively (Fig. 1B). No further stabilization was observed in a *ubc6 Δ ubc7 Δ* double deletion as compared with the *ubc7 Δ* single deletion, consistent with findings for *Mata2* turnover (33). In agreement with results published previously (17), we found that Ste6p* was also strongly stabilized in the absence of Cue1p, an ER membrane protein



that binds Ubc7p and positions it at the ER membrane.² Thus, the E2 requirements for Ste6p* ERAD are similar to those reported for soluble proteins with the *Mata2*-derived *Deg1* degradation signal (33) and for Ubc6p, itself an ERAD substrate (34).

Two E3 ubiquitin ligases are known to be involved in the ERAD pathway, Hrd1p (also called Der3p) and Doa10p (35–37). Hrd1p acts in a complex with Hrd3p, along with the E2 enzymes Ubc7p and Ubc1p, and is required for the degradation of many ERAD substrates, including CPY*, Hmg2p, Pdr5p*, and Sec61-2p (12, 38, 39). Doa10p was discovered in a screen for mutants that stabilize the transcriptional repressor *Mata2* (37), and also functions in the ERAD of a mutant form of Pma1p (D378N) (40) and in the constitutive turnover of wild-type Ubc6p (37). It has been suggested that Doa10p functions with both Ubc6p and Ubc7p, whereas Hrd1p action is primarily Ubc6p-independent (8). As both Ubc6p and Ubc7p appear to contribute to the degradation of Ste6p*, we suspected that Doa10p might be the E3 ligase for Ste6p*. Indeed, when we examined Ste6p* turnover in *hrd1 Δ* or *hrd3 Δ* deletion strains, we observed little or no effect, whereas Ste6p* was strongly stabilized (~ 4 -fold) in a *doa10 Δ* mutant (Fig. 2, A and C). The opposite E3 ligase dependence was observed for CPY* (Fig. 2B), as reported previously (37, 38). Interestingly, when *HRD1* was also deleted in a *doa10 Δ* background, an additional but small increase in Ste6p* was consistently observed (Fig. 2C), indicat-

² G. Huyer and S. Michaelis, unpublished observations.

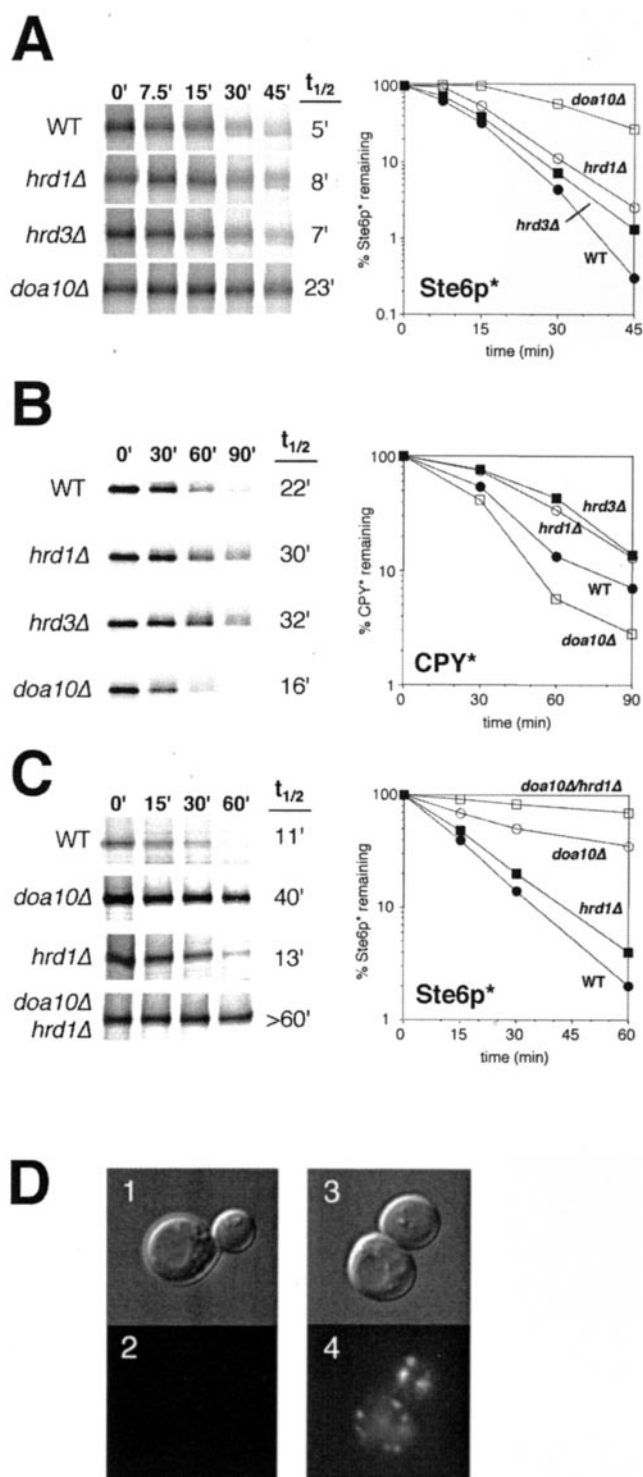


FIG. 2. Distinct E3 ubiquitin ligases facilitate the ERAD of Ste6p* and CPY*. Turnover of Ste6p* (A and C) and CPY* (B) was examined by pulse-chase analysis as described in the legend to Fig. 1. Strains used were BY4741 (WT), SM4823 (*hrd1Δ*), SM4824 (*hrd3Δ*), and SM4820 (*doa10Δ*) transformed with either pSM1694 (Ste6p*-HA-GFP) or pSM1763 (CPY*-HA) (A and B), and MHY500 (WT), MHY1685 (*doa10Δ*), MHY2822 (*hrd1Δ*), and MHY1702 (*doa10Δ hrd1Δ*) transformed with pSM1082 (Ste6p*-HA) (C). D, localization of Ste6p* in the absence of Doa10p. The corresponding differential interference contrast (top) and GFP (bottom) images are shown for wild-type (BY4741, panels 1 and 2) and *doa10Δ* (SM4820, panels 3 and 4) expressing Ste6p*-HA-GFP (pSM1694).

ing that Hrd1p can partially compensate if Doa10p is absent. Therefore, more than one ER-embedded ubiquitin ligase appears to be able to recognize the same ER membrane protein

substrate, although Doa10p is significantly more active on Ste6p* than Hrd1p.

We also examined the localization of GFP-tagged Ste6p* in wild-type and *doa10Δ* strains by fluorescence microscopy. Not surprisingly, we were unable to observe Ste6p*-GFP in wild-type cells by fluorescence microscopy, consistent with its rapid turnover (Fig. 2D, panel 2). Most interestingly, in the *doa10Δ* mutant, we observed Ste6p* primarily in ERACs, a quality control subcompartment of the ER induced by Ste6p* (and certain other mutant forms of Ste6p (22)) (Fig. 2D, panel 4). ERAC localization of Ste6p* was confirmed by colocalization with the ER marker Sec63p-RFP.² Thus, the formation of ERACs and the segregation of Ste6p* into these subcompartments must occur prior to ubiquitination, suggesting the existence of factors upstream of the ubiquitination machinery required for recognition of Ste6p*.

A Role for Cytosolic Chaperones in ERAD of Ste6p*—The luminal ER chaperone Kar2p plays a role in the ERAD of CPY* (41), presumably by recognizing the misfolded protein and retaining it in an unfolded conformation prior to retrotranslocation (42). However, because of the largely cytosolic orientation of Ste6p* and the cytosolic location of the Q1249X mutation (Fig. 1A), we predicted that Kar2p should not be involved in Ste6p* turnover. Indeed, we observed no stabilization of Ste6p* at the nonpermissive temperature in the *kar2-133* mutant, whereas CPY* was stabilized ~2-fold (Fig. 3A).

We next examined the role of cytosolic chaperones in Ste6p* degradation. We showed previously (43) that the cytosolic Hsp70, Ssa1p, functions in ERAD of CFTR expressed in yeast, consistent with the predominantly cytosolic topology of this multispansing membrane protein. When we compared Ste6p* and CPY* turnover in an *ssa1-45* temperature-sensitive strain at the nonpermissive temperature, we observed that degradation of Ste6p* was inhibited greater than 2-fold, whereas CPY* stability was unaffected (Fig. 3B). Thus, consistent with their differing topologies and site of mutation, Ste6p* and CPY* require different Hsp70 chaperones for their efficient degradation, providing evidence that these two classes of proteins follow distinct degradation pathways.

The function of Hsp70 chaperones depends on co-chaperones of the DnaJ (Hsp40) family to stimulate their ATPase activity, and in some cases for the delivery of polypeptide substrates (44). In the case of Kar2p, different DnaJ homologues appear to provide functional specificity, with Sec63p coupling with Kar2p during ER translocation, and Scj1p and Jem1p acting in conjunction with Kar2p during the ERAD of CPY* (42). To explore if Ssa1p similarly cooperates with a DnaJ homologue in the ERAD of Ste6p*, we sought to determine which cytosolic DnaJ homologue(s) may be required. Ydj1p is a known co-chaperone for Ssa1/2p (45) that is tethered to the ER membrane by prenylation (46), and Hlj1p is a C-terminally anchored ER membrane protein with homology to DnaJ (47) that has recently been found to stimulate the ATPase activity of Ssa1p.³ In a strain bearing a temperature-sensitive allele of *YDJ1* (*ydj1-151*) and a deletion of *HLJ1*, Ste6p* degradation was strongly inhibited (Fig. 4A). In strains bearing single mutations of the co-chaperones, we observed little or no stabilization of Ste6p*,² suggesting that Ydj1p and Hlj1p function redundantly with Ssa1p to facilitate Ste6p* degradation. A similar result with the single and double mutants has been observed for degradation of CFTR expressed in yeast.³ Consistent with Ssa1p being dispensable for CPY* degradation, mutation of *YDJ1* and *HLJ1*, either singly or together, had no effect on CPY* turnover (Fig. 4B).²

³ Youker, R. T., Walsh, P., Beilharz, T., Lithgow, T., and Brodsky, J. L. (2004) *Mol. Biol. Cell*, in press.

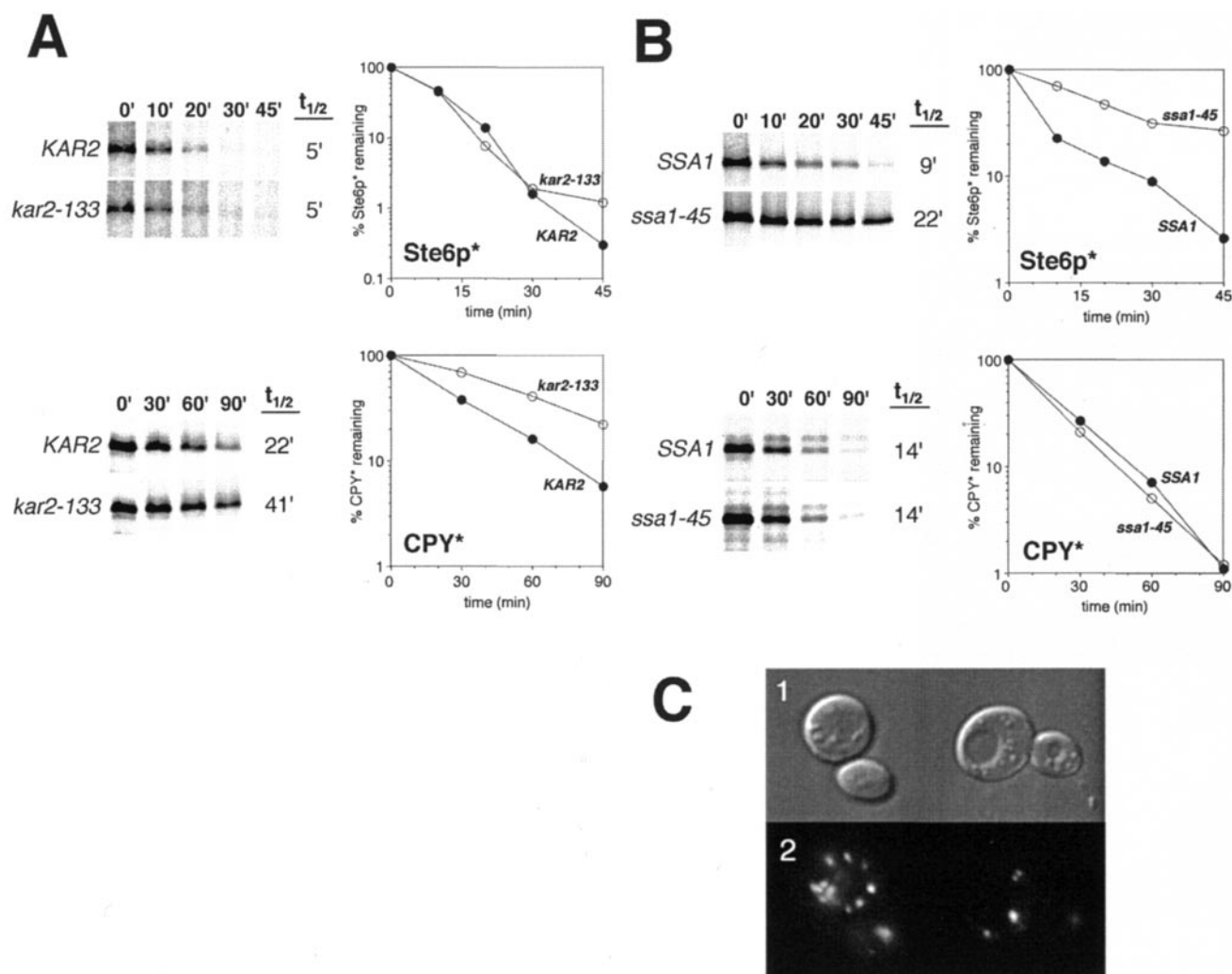


FIG. 3. Differential Hsp70 chaperone requirements for ERAD of Ste6p* and CPY*. A, turnover of CPY* but not Ste6p* is facilitated by the ER luminal Hsp70 Kar2p. Pulse-chase experiments were conducted as described in the legend to Fig. 1, with cells grown at room temperature and then shifted to 37 °C for 10 min prior to labeling. Strains used were MS10 (*KAR2*) and MS193 (*kar2-133*) expressing Ste6p*-HA-GFP (pSM1694) or CPY*-HA (pSM1763). B, turnover of Ste6p* but not CPY* is facilitated by the cytosolic Hsp70 Ssa1p. Pulse-chase experiments were conducted as described in the legend to Fig. 1, with cells grown at room temperature and then shifted to 37 °C for 10 min prior to labeling. Strains used were SM4247 (*SSA1*) and SM4177 (*ssa1-45*) expressing Ste6p*-HA (pSM1911) or CPY*-HA (pSM1763). C, localization of Ste6p* in the *ssa1-45* strain. The corresponding differential interference contrast (panel 1) and GFP (panel 2) images are shown for *ssa1-45* (SM4177) expressing Ste6p*-HA-GFP (pSM1912). Cells were grown to mid-logarithmic phase at room temperature and then shifted to 37 °C for 45 min before viewing.

We also examined the localization of GFP-tagged Ste6p* in the *ssa1-45* and *ydj1-151 hlj1*Δ mutant strains. As we observed for the *doa10*Δ mutant, Ste6p*-GFP was found in ERACs in these chaperone mutant strains (Fig. 3C).² This localization suggests that cytosolic chaperones function after Ste6p* is segregated into ERACs and that they are not involved in the initial recognition of Ste6p*. Furthermore, we did not observe GFP-tagged Ste6p* in the cytosol when the chaperones were mutated, indicating that the cotranslational insertion of Ste6p* into the membrane is unaffected.

*The Sec61p Translocon Does Not Appear to Be Required for ERAD of Ste6p**—An apparent conundrum in ERAD is how ER luminal and membrane proteins gain access to the cytosolic proteasome for degradation. An answer was provided with the discovery of a role for the mammalian Sec61 translocon pore in transfer of a type I transmembrane protein from the ER to the proteasome (48). Studies in yeast provided further evidence for retrotranslocation of ERAD substrates being dependent on the Sec61p translocon pore, based on the isolation of *sec61* alleles that block the export (retrotranslocation) of ERAD substrates from the ER and into the cytosol (41, 49, 50). Thus, luminal ERAD substrates like CPY* are stabilized in these mutants

because they cannot gain access to the cytosolic proteasome. However, whether or not yeast membrane proteins are retrotranslocated through the Sec61p pore prior to ERAD is currently a subject of debate. In particular, the multispanning membrane protein Pdr5p* (a mutant form of Pdr5p) is stabilized in a retrotranslocation-defective *sec61-2* mutant (39), whereas degradation of the C-terminally anchored, type I membrane protein Ubc6p is unaffected in three retrotranslocation-defective *sec61* alleles (34).

To determine whether retrotranslocation is required for ERAD of Ste6p*, we examined its turnover in a *sec61-2* mutant that is strongly defective for retrotranslocation, but only modestly defective for forward translocation at the permissive temperature (24 °C) (51). Similar to previously reported studies (34, 39), CPY* was stabilized ~2-fold (Fig. 5A). However, there was no effect on Ste6p* turnover (Fig. 5A), suggesting that Ste6p* degradation is independent of Sec61p function.

It has been proposed that Ssh1p, a homologue of Sec61p, may function as an alternate pore for CPY* retrotranslocation (52). However, the role of Ssh1p is complicated for two reasons. First, it is also required for translocation of CPY* into the ER. Second, a respiratory-deficient (*rho*-minus) *ssh1*Δ mutant does

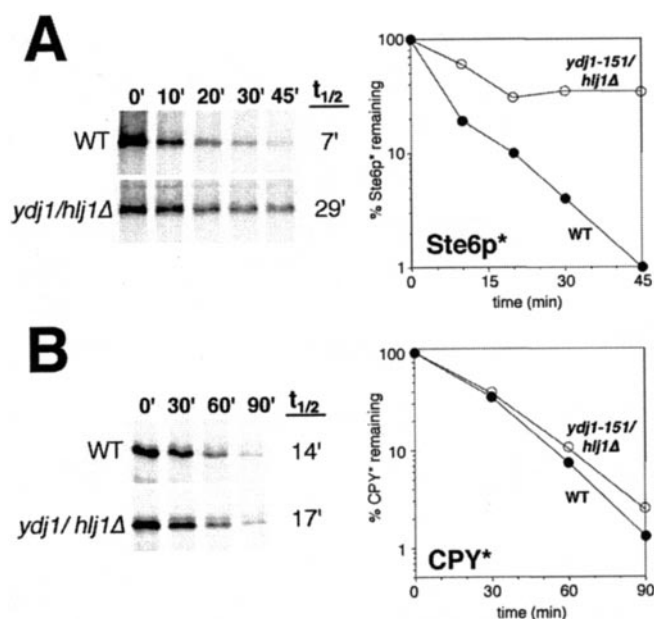


FIG. 4. The cytosolic Hsp40s Ydj1p and Hlj1p play redundant roles in ERAD of Ste6p*. Turnover of Ste6p* (A) and CPY* (B) was examined by pulse-chase analysis as described in the legend to Fig. 1, with cells grown at room temperature and then shifted to 37 °C for 10 min prior to labeling. Strains used were SM4947 (WT) and SM4949 (*ydj1-151 hlj1Δ*) transformed with either pSM1911 (Ste6p*-HA) or pSM1763 (CPY*-HA).

not stabilize CPY* (52). Here we examined whether Ssh1p plays a role in Ste6p* turnover and obtained conflicting results. First, we observed no stabilization of either Ste6p* or CPY* in three different respiratory-competent strain backgrounds (BY4741, W303, and YJL183) containing an *ssh1* deletion, grown in glucose (Fig. 5B).² However, with the same *ssh1Δ* strain and media conditions used by Wilkinson *et al.* (52) (*i.e.* grown in glycerol/ethanol and then shifted to glucose for 5 h before labeling), we observed almost complete stabilization of Ste6p* as well as the previously reported effect on CPY* translocation and turnover (Fig. 5C). Finally, we re-examined the BY4741 strain containing the *ssh1Δ* deletion under the same media conditions (glycerol/ethanol and then glucose) as described by Wilkinson *et al.* (52) and still found no effect on CPY* turnover, but Ste6p* was now stabilized more than 3-fold.² Because of the strain background variability (which may suggest secondary mutations) and different effects of carbon source (indicative of physiological influences), it is difficult to conclude that Ssh1p acts as an alternative retrotranslocon for Ste6p*. Instead, it may affect Ste6p* degradation indirectly under some conditions. These conflicting results suggest that further genetic and biochemical analyses will be required to clarify the role (if any) of Ssh1p in ERAD.

ERAD Pathways for Ste6p* and CPY* Converge after Ubiquitination but before the Proteasome—Cdc48p, a member of the AAA family of ATPases, has been shown to function in a complex with Npl4p and Ufd1p at a post-ubiquitination step in the ERAD of multiple substrates (53–57). The complex was suggested to assist in the recruitment of the proteasome (58) and/or in the translocon-dependent dislocation and disaggregation of ERAD substrates through its ability to bind to ubiquitinated and nonubiquitinated proteins (59). As Ste6p* ERAD does not appear to require Sec61p function, we were unsure whether there would be a requirement for the Cdc48p complex in Ste6p* degradation. Interestingly, when we examined the effect of the temperature-sensitive *cdc48-3* mutant on Ste6p* turnover, we found that Ste6p* was strongly stabilized (~4-fold) at the nonpermissive temperature (Fig. 6A). We also ex-

amined the localization of Ste6p* in the *cdc48-3* mutant. As for the *doa10Δ* and *ssa1-45* mutants (Figs. 2D and 3C), Ste6p*-GFP accumulated in ERACs (Fig. 6B), a localization pattern that is consistent with Cdc48p acting after Ste6p* is segregated into ERACs.

DISCUSSION

Ste6p*, a Model Protein for the ERAD of Multispanning Transmembrane Proteins—In this study, we use Ste6p* to investigate ER quality control of a multispanning membrane protein. As compared with other membrane proteins used to study ERAD in yeast, Ste6p* (Ste6p-Q1249X) has the important advantages that Ste6p is an endogenous yeast protein and that the Q1249X mutation lies in a topologically defined cytosolic domain. Most of the membrane proteins that have been studied as ERAD substrates in yeast, including Hmg2p (60), Ubc6p (34), Vph1p in the absence of Vma22p (61), and heterologously expressed human CFTR (43), are wild-type proteins in which the locations of the misfolded domains are unknown. In two recent studies, ERAD substrates with topologically defined mutations have been created by fusing folded and misfolded domains to a transmembrane span (20, 62), but these are artificial constructs. As a *bona fide* mutated yeast protein, Ste6p* represents a valuable model protein to probe the requirements for ERAD of an endogenous multispanning membrane protein with a mutation in a cytosolic domain.

Our studies with Ste6p* show that its ERAD pathway has significant differences from that of the soluble luminal protein CPY*, as summarized in Fig. 7. The degradation pathways of both substrates converge after ubiquitination, with overlapping requirements for Cdc48p and the proteasome. However, the ubiquitination machinery differs as follows: Ste6p* degradation is dependent primarily on the E3 ligase Doa10p, whereas CPY* degradation is dependent on the E3 ligase Hrd1p/Der3p. Both substrates require the E2 Ubc7p but Ste6p* also requires Ubc6p, as was observed with other Doa10p substrates (33, 34). Interestingly, Ste6p* ERAD does not appear to require the Sec61p translocon pore, in contrast to CPY*, suggesting that Ste6p* is retrotranslocated by an alternative mechanism or possibly is degraded without extraction of the full-length polypeptide from the membrane. Finally, Ste6p* and CPY* exhibit differential chaperone requirements for their degradation, with Ste6p* dependent on cytosolic chaperones (the Hsp70 Ssa1p and the Hsp40 Ydj1p and Hlj1p) and Kar2p dependent on a luminal one (the Hsp70 Kar2p). The significance of these findings is discussed in detail below.

Distinct Machinery Required for Ubiquitination of Ste6p* and CPY*—Ubiquitination is an important signal not only in ERQC but also in endocytosis, and in both processes the substrate specificity is determined at least in part by E3 ubiquitin ligases. For wild-type Ste6p and other endocytosed proteins, ubiquitination acts as a signal to direct internalization and targeting to the vacuole via the multivesicular body (reviewed in Refs. 63 and 64). The ubiquitination of endocytic substrates is believed to be catalyzed by the E3 ligase Rsp5p in conjunction with the E2 ubiquitin-conjugating enzymes Ubc4p and Ubc5p, and degradation is dependent on vacuolar proteases. In ERQC, ubiquitination also plays a critical role in targeting proteins, but in this case ubiquitination occurs at the ER, and degradation is mediated by the cytosolic proteasome.

In this study we show that for Ste6p*, the principal E3 ubiquitin ligase required for degradation is Doa10p (Fig. 2A), as has also been reported recently by others (20), although Hrd1p can partially compensate if Doa10p is absent (Fig. 2C). Doa10p was discovered based on its requirement for degradation of the transcriptional repressor Mata2 (and *Deg1*-containing derivatives) (37); other substrates include the type I mem-

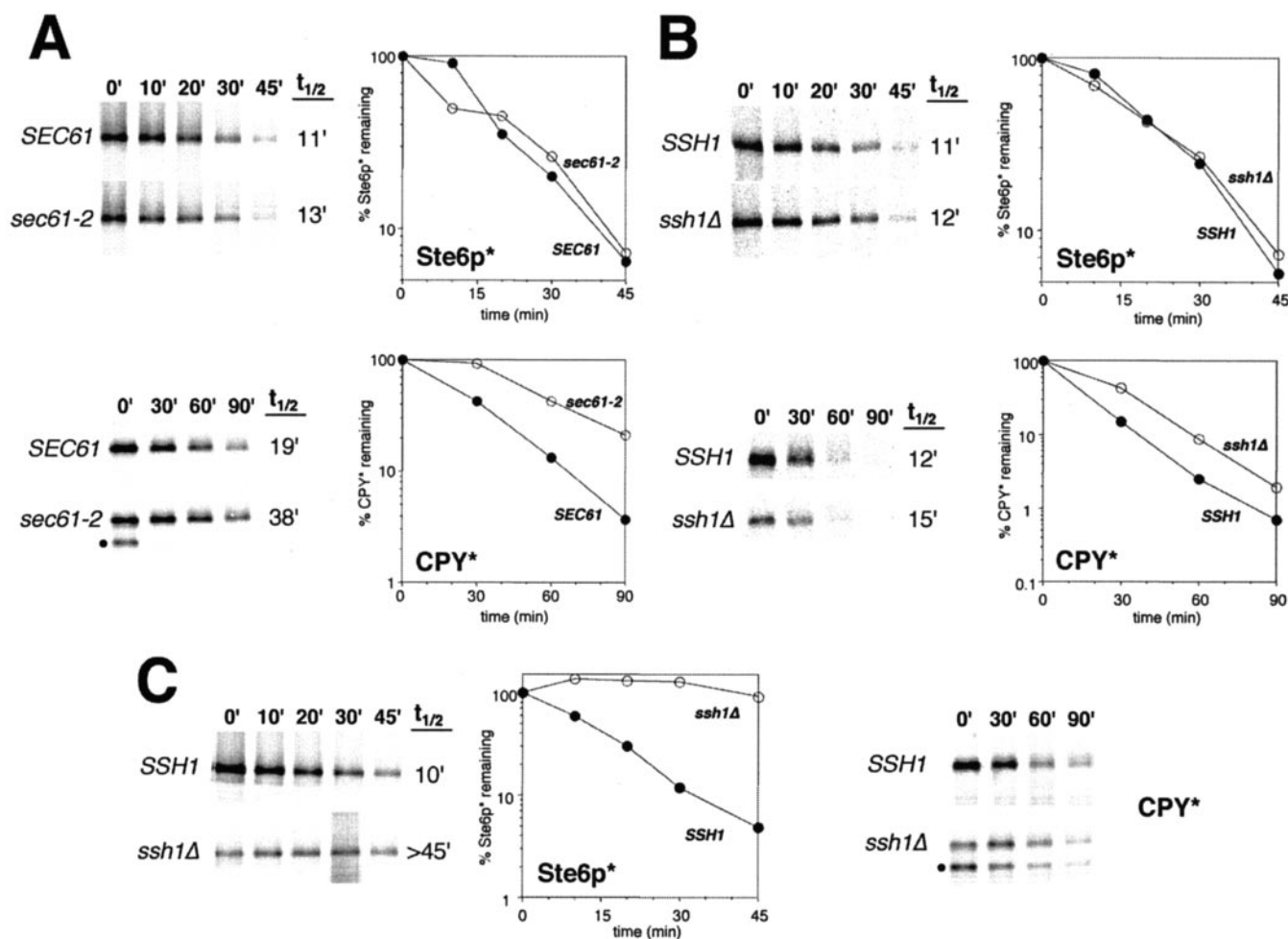


FIG. 5. ERAD of Ste6p* does not appear to require retrotranslocation through the Sec61p translocon but could depend on Ssh1p. A, turnover of Ste6p* and CPY* was examined by pulse-chase analysis as described in the legend to Fig. 1, with cells grown and labeled at room temperature. For CPY*, the lower band in the sec61-2 pulse-chase at 0 min (●) represents unglycosylated prepro-CPY* that accumulates in the cytosol because of a slight delay in translocation (34). Strains used were RSY156 (SEC61) and RSY533 (sec61-2), transformed with either pSM1911 (Ste6p*-HA) or pSM1763 (CPY*-HA). B, turnover of Ste6p* and CPY* was examined by pulse-chase analysis as described in the legend to Fig. 1. Strains used were BY4741 (SSH1) and SM5129 (ssh1Δ) transformed with pSM1694 (Ste6p*-HA-GFP) or pSM1763 (CPY*-HA). C, turnover of Ste6p* and CPY* was examined by pulse-chase analysis as described in the legend to Fig. 1, except that the cells were grown in media containing 3% glycerol and 0.3% ethanol instead of glucose as the carbon source and then shifted to media containing glucose for 5 h before metabolic labeling, as described (52). The lower band in the CPY* ssh1Δ pulse-chase (●) is prepro-CPY*. Strains used were BWY46 (SSH1) and BWY464 (ssh1Δ) transformed with pSM1911 (Ste6p*-HA) or pSM1763 (CPY*-HA). It was not possible to quantitate accurately the CPY* turnover because of the defect in translocation into the ER, so no quantitation graph is shown for CPY*.

brane protein Ubc6p (37) and a mutant form of the multispanning membrane protein Pma1p (40). In contrast, a different E3, namely Hrd1p/Der3p, is required for degradation of the luminal substrate CPY*, as well as for the multispanning membrane proteins Hmg2p, Pdr5p*, and Sec61-2p (12, 38, 39). It is unclear what distinguishes Doa10p and Hrd1p substrates. The identification of additional substrates for these E3 ligases and determination of their substrate binding domains may help to clarify the substrate specificity differences. Subcellular localization may also influence specificity. For example, Doa10p may reside in the inner nuclear membrane in addition to the ER membrane, where it would have access to the nuclear protein Mata2, although Mata2 could also exit the nucleus via nuclear pores to gain access to Doa10p at the ER membrane (37). Interestingly, the increased stabilization of Ste6p* we observed in a doa10Δ hrd1Δ double mutant (Fig. 2C), as compared with a doa10Δ single mutant, suggests that there is some overlap in substrate specificity of these E3 ligases.

Notably, we found here that both the Ubc6p and Ubc7p E2 ubiquitin-conjugating enzymes are required for the degradation of Ste6p*. Doa10p has been proposed to act in a complex

with both Ubc6p and Ubc7p, based on the observation that the degradation of the Doa10p substrates Mata2 and Ubc6p is equally dependent on both E2s (37). Our results with Ste6p* suggest that both Ubc6p and Ubc7p will generally participate in the degradation of Doa10p substrates.

We also examined the localization of components of the ERAD machinery, namely Doa10p and Ubc7p (expressed chromosomally as C-terminal GFP fusions), in strains expressing Ste6p*. Ste6p* and other mutant forms of Ste6p that are ERAD substrates induce the formation of, and localize exclusively to, ERACs, a quality control subcompartment of the ER (22), and an interesting possibility would be that the ERAD machinery for Ste6p* is specifically concentrated in ERACs. However, as we have observed for other ER resident proteins (Ste24p, Ste14p, and Sec63p (22)), Doa10p and Ubc7p were present both in ERACs and in the ER, with no obvious concentration in ERACs or depletion from the ER.² Studies are ongoing in our laboratory to identify factors that are enriched in ERACs.

ERAD of Ste6p* Depends on Cytosolic, Not ER-luminal, Chaperones—The heat shock-inducible proteins of the Hsp70 family and the constitutively expressed heat shock cognate

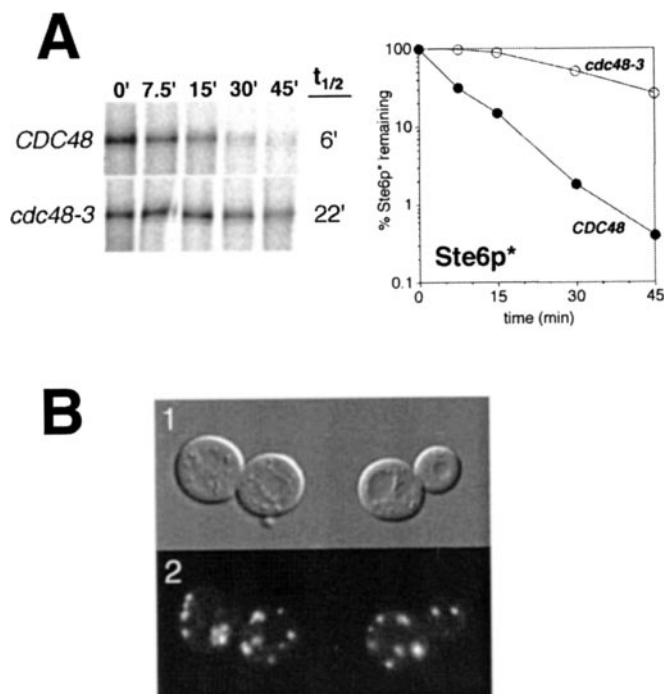


FIG. 6. ERAD of *Ste6p is facilitated by the ATPase *Cdc48p*.** *A*, turnover of *Ste6p** was examined by pulse-chase analysis as described in the legend to Fig. 1, with cells grown at room temperature and then shifted to 37 °C for 10 min prior to labeling. Strains used were W303a (*CDC48*) and SM4783 (*cdc48-3*), transformed with pSM1694 (*Ste6p**-HA-GFP). *B*, localization of *Ste6p** in the *cdc48-3* strain. The corresponding differential interference contrast (panel 1) and GFP (panel 2) images are shown for *cdc48-3* (SM4783) expressing *Ste6p**-HA-GFP (pSM1694). Cells were grown to mid-logarithmic phase at room temperature and then shifted to 37 °C for 60 min before viewing.

(Hsc70) chaperones play critical roles in the proper folding of proteins both in the cytosol and the ER lumen and in catalyzing protein import into the ER (reviewed in Ref. 44). Not unexpectedly, chaperones also function in ER quality control, presumably by recognizing the misfolded protein substrates and assisting their unfolding to facilitate ERAD (44). The topology of *Ste6p** suggests that cytosolic rather than luminal chaperones would be involved in its degradation, as very little of *Ste6p** resides in the ER lumen, and the site of mutation is in the cytosol (Fig. 1A). Consistent with this prediction, the luminal Hsp70 *Kar2p* is not required for the ERAD of *Ste6p**, whereas the cytosolic Hsp70, *Ssa1p*, is required (Fig. 3). Similar results have been observed with two other multispanning membrane proteins with predominantly cytosolic domains, namely CFTR expressed in yeast (43) and unassembled *Vph1p* subunits (61), whereas *CPY** degradation requires *Kar2p* function and not *Ssa1p* (Fig. 3) (41).

We further demonstrated that two ER-associated Hsp40 co-chaperones, namely *Ydj1p* and *Hlj1p*, are also required for ERAD of *Ste6p** but not *CPY** (Fig. 4). Hsp40s enhance cognate Hsp70 ATPase activity and substrate trapping and also can bind directly to misfolded proteins, which may contribute to the specificity of chaperone-substrate interaction (44). Based on the data presented here, we suggest that the association of *Ssa1p* with either *Ydj1p* or *Hlj1p* results in *Ssa1p* recruitment to the cytosolic face of the ER membrane from the cytosol, in proximity to ERQC substrates like *Ste6p**.

Importantly, the differential chaperone dependence of *Ste6p** and *CPY** suggests that ERAD substrates should be distinguished not on the basis of their being ER luminal or membrane proteins but rather on the topological location of the misfolded domain. Specifically, *Ste6p**, a multispanning mem-

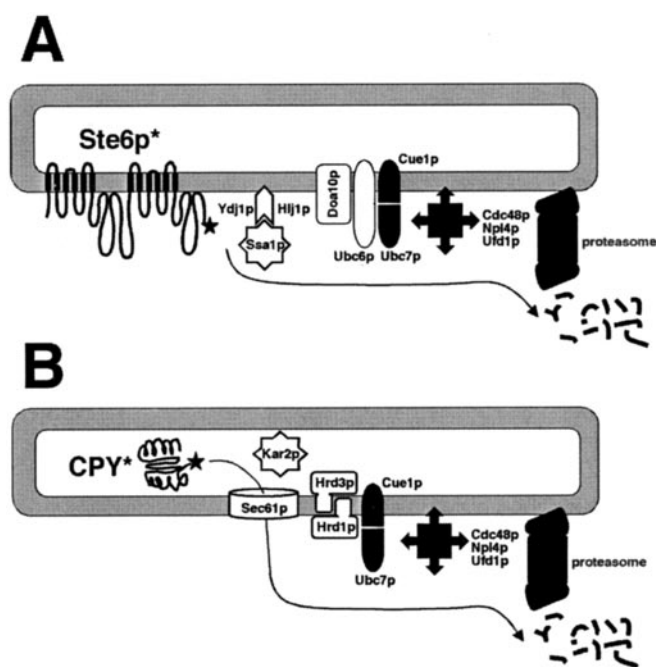


FIG. 7. ERAD pathways for *Ste6p and *CPY**.** Working models for *Ste6p** and *CPY** ERAD are depicted, based on the results of this study and elsewhere. The *star* in the *Ste6p** and *CPY** protein schematics represents the misfolded domain. *A*, *Ste6p** is ubiquitinated by the E3 ligase *Doa10p* in conjunction with the E2 enzymes *Ubc6p* and *Ubc7p*. The cytosolic Hsp70 *Ssa1p* and its co-chaperones, *Ydj1p* and *Hlj1p*, are drawn between *Ste6p** and the ubiquitination machinery; however, it has not been determined if the chaperones act before or after ubiquitination and therefore the placement is arbitrary. The ATPase *Cdc48p* acts in a complex with *Npl4p* and *Ufd1p* at a post-ubiquitination step but prior to degradation by the proteasome. It is unclear if *Ste6p** is extracted from the membrane prior to degradation, as the *Sec61p* translocon pore does not appear to be required for its ERAD. *B*, recognition of *CPY** for ERAD may involve *Hrd3p* and *Kar2p*, acting either coordinately or independently. *Kar2p* may retain *CPY** in an unfolded conformation to permit retrotranslocation through the *Sec61p* pore, bringing *CPY** in contact with the ubiquitination machinery (*Hrd1p* and *Ubc7p*) on the cytosolic face of the ER membrane. As is the case for *Ste6p**, the *Cdc48p* complex assists in delivery of ubiquitinated *CPY** to the proteasome for degradation. *A* and *B*, machinery in *black* has been demonstrated here and elsewhere to be used in common for the ERAD of *Ste6p** and *CPY** (the E2 ubiquitin-conjugating enzyme *Ubc7p* and its associated protein *Cue1p*, the complex containing the ATPase *Cdc48p*, and the proteasome), whereas distinct machinery is depicted in *white* (the Hsp70 chaperones *Kar2p* and *Ssa1p*, the Hsp40 co-chaperones *Ydj1p* and *Hlj1p*, the *Sec61p* translocon, the E2 *Ubc6p*, and the E3 ubiquitin ligases *Doa10p* and *Hrd1p* in a complex with *Hrd3p*). All the ERAD machinery (except the chaperone *Kar2p*) is placed at the ER membrane, either because they are integral ER membrane proteins (*Hlj1p*, *Doa10p*, *Hrd1p*, *Hrd3p*, and *Ubc6p*) or because they can associate with the ER membrane directly (*Ydj1p*, *Cdc48p* complex, and proteasome) or indirectly through another ER membrane protein (*Ubc7p* with *Cue1p*, *Ssa1p* with *Ydj1p*, and *Hlj1p*).

brane protein with a cytosolic misfolded domain, depends upon cytosolic chaperones for its degradation, whereas *CPY**, a soluble luminal protein, depends upon luminal chaperones for its degradation. Indeed, it has been demonstrated recently (20) that a membrane protein with a misfolded luminal domain requires similar machinery to an ER luminal substrate. Because of the importance of the topological site of the misfolded domain in defining ERAD pathways, an ERAD substrate like *Ste6p** with a mutation in a defined domain may provide certain advantages as a model substrate compared with a protein with an undefined site of misfolding.

*Translocon-independent Degradation of *Ste6p***—It is particularly interesting that the *Sec61p* translocon pore may not be involved in ERAD of *Ste6p**, as demonstrated by a lack of stabilization of *Ste6p** in the *sec61-2* mutant (Fig. 5A). Because

insertion of multispinning membrane proteins into the ER membrane requires the lateral release of membrane spans into the lipid bilayer from the pore, it has been proposed that multispinning membrane proteins are removed in a reverse manner (reviewed in Refs. 65 and 66). In yeast, the Sec61p translocon has been shown to be required for ERAD of the multispinning membrane protein Pdr5* (39), but it is not required for the degradation of the type I membrane protein Ubc6p (34). Genetic evidence suggests that Hrd1p/Der3p and Hrd3p interact with Sec61p, perhaps forming a functional complex for ERAD of certain ERQC substrates (67). Thus, Hrd1p substrates may be retrotranslocated through the Sec61p pore, whereas Doa10p substrates may be dislocated by another mechanism. In accordance with this model, the ERAD substrates that have been shown to be dependent on Sec61p (*i.e.* CPY* (41) and Pdr5* (39)) are Hrd1p substrates, whereas those that appear to be retrotranslocation-independent (*i.e.* Ubc6p (34) and Ste6p* (this study)) are Doa10p substrates. In fact, it has been proposed that Doa10p itself may form a kind of retrotranslocation channel through its multiple (10 to 14) transmembrane domains, providing an alternative mode for dislocation of membrane proteins (37). Indeed, other factors are likely to be involved in dislocation of ERAD substrates, as suggested by the recent discovery of Derlin-1, proposed to be part of a novel retrotranslocation channel (68, 69). However, the yeast Derlin-1 homologue, Der1p, is not required for Ste6p* ERAD (20).

Based on our studies, it is also possible that Ssh1p could play a direct or indirect role in Ste6p* dislocation. Ssh1p was discovered as a ribosome-binding protein, suggesting that its main role is in cotranslational translocation into the ER (70); nevertheless, Ssh1p has been proposed to function as part of an alternative retrotranslocon for CPY* (52). Our analysis of both Ste6p* and CPY* turnover in the absence of Ssh1p was inconclusive, with stabilization observed in only one of four *ssh1Δ* strain backgrounds examined and influenced by the choice of media and carbon source (Fig. 5, B and C).² Additional study will be required to determine whether Ssh1p is indeed functioning in the retrotranslocation of Ste6p* and/or CPY* or if its role in the degradation of ERAD substrates is indirect.

One might ask whether Ste6p* even needs to be extracted from the membrane prior to degradation. It is possible that Ste6p* simply remains in the ER membrane, in which case the proteasome may shave off the cytosolic loops by endoproteolytic cleavage, with the residual membrane and luminal fragments being degraded by other proteases or extracted by the proteasome independent of Sec61p (or Ssh1p) function. The proteasome appears to be capable of endoproteolytic cleavage, based on its ability to release the membrane-bound transcription factors Spt23p and Mga2p (71–73) and to cleave at internal sites in model substrates lacking accessible termini (74, 75). Recruitment of the cytosolic proteasome to ubiquitinated Ste6p* could be mediated by the Cdc48p-Ufd1p-Npl4p complex, as this complex is capable of binding both the 19 S “cap” of the proteasome and ubiquitinated proteins (via Cdc48p) and also associates with the ER membrane (via Npl4p) (58). We are currently investigating whether degradation intermediates of Ste6p* can be observed that would be consistent with removal of its cytosolic loops.

Other Unanswered Questions—An important question that remains is how proteins are recognized and targeted for ERAD. Proteins with luminal lesions may follow a distinct pathway from those with cytosolic lesions (referred to as ERAD-L and ERAD-C, respectively (20)). However, the recognition machinery is not well understood. In the case of Pma1p-D378N, an ERAD substrate, Eps1p appears to function as a recognition

factor for the mutant Pma1p protein, as Eps1p directly interacts with Pma1p-D378N (but not wild-type Pma1p), and the mutant Pma1p protein is no longer retained in the ER when Eps1p is absent (40). We asked whether Eps1p may also recognize Ste6p* for ERAD, by examining Ste6p* turnover in three different strain backgrounds (BY4741, our laboratory strain background SM1058, and in the original strain used by Wang and Chang (40)). In each case we observed no stabilization of Ste6p*,² suggesting that Eps1p is specific for the degradation of a Pma1p mutant protein.

Recognition also involves a direct interaction of the E3 ubiquitin ligase with its substrate, as has been elegantly demonstrated with Hrd1p and Hmg2p (60). In the case of Ste6p*, it is likely that the E3 ubiquitin ligase Doa10p, and perhaps Hrd1p, participates in recognition. However, there must be an additional earlier recognition step for Ste6p* that leads to its induction of and segregation into ERACs, because Ste6p* is found in ERACs when *DOA10* is deleted (Fig. 2D). Similarly, mutation of the cytosolic chaperones Ssa1p, Ydj1p, and Hlj1p does not prevent sorting of Ste6p* into ERACs (Fig. 3C),² indicating that these chaperones are also not involved in this early recognition step. It will be of great interest to determine what factor(s) are required for this initial recognition and sorting step, as therapeutic inhibition of such a factor may permit partially functional ERAD substrates, like CFTR-ΔF508 (a mutant disease-causing allele of CFTR), to escape degradation and help alleviate the associated disease symptoms.

In summary, we have demonstrated that Ste6p* is a valuable model protein for studying the ERAD of multispinning membrane proteins. The quick turnover ($t_{1/2} \sim 10$ min) of Ste6p* greatly facilitates analysis of its degradation by pulse-chase studies, and when it is expressed as a GFP fusion its localization can easily be evaluated in mutants that stabilize the protein. We expect that these and other investigations with Ste6p* will continue to reveal novel components and interesting new features of ER quality control of multispinning membrane proteins, with implications for our understanding mammalian ER quality control and the many diseases connected with this system.

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