0026-895X/06/6906-1897–1904$20.00
MOLECULAR PHARMACOLOGY
Vol. 69, No. 6
21816/3118271
Copyright © 2006 The American Society for Pharmacology and Experimental Therapeutics
*Mol Pharmacol* 69:1897–1904, 2006
*Printed in U.S.A.*

# Endoplasmic Reticulum-Associated Degradation of Cytochrome P450 CYP3A4 in *Saccharomyces cerevisiae*: Further Characterization of Cellular Participants and Structural Determinants

Mingxiang Liao, Saadia Faouzi, Andrey Karyakin, and Maria Almira Correia

*Departments of Cellular and Molecular Pharmacology, Pharmaceutical Chemistry, and Biopharmaceutical Sciences and the Liver Center, University of California, San Francisco, California*

Received December 18, 2005; accepted March 23, 2006

**ABSTRACT**

The monotopic, endoplasmic reticulum (ER)-anchored cytochromes P450 (P450s) undergo variable proteolytic turnover. CYP3A4, the dominant human liver drug-metabolizing enzyme, is degraded via a ubiquitin (Ub)-dependent 26S proteasomal pathway after heterologous expression in *Saccharomyces cerevisiae*. This turnover involves the Ub-conjugating enzyme Ubc7p and the 19S proteasomal subunit Hrd2p but is independent of Hrd1p/Hrd3p, a major Ub-ligase (E3) involved in ER protein degradation. We now show that CYP3A4 ERAD also involves the Ubc7p-ER anchor Cue1p, because CYP3A4 is significantly stabilized at the stationary growth phase in Cue1p-deficient yeast. To determine whether the other major Ub-ligase Doa10p or Rsp5p involved in ER protein degradation functions in CYP3A4 ERAD, wild type and Doa10p- or Rsp5p-deficient yeast strains were also similarly examined. No appreciable CYP3A4 stabilization was detected in either Doa10p- or Rsp5p-deficient yeast, thereby excluding these E3s and revealing that CYP3A4 ERAD involves a novel or yet to be identified E3. Similar studies also revealed that the Cdc48p-Ufd1p-Hrd4p complex, responsible for the translocation of polyubiquitinated ER proteins was critical for CYP3A4 ERAD. We previously reported that grafting of the C-terminal (CT) CYP3A4 heptapeptide onto the CYP2B1 C terminus switched its proteolytic susceptibility from predominantly vacuolar to proteasomal degradation. To determine the relevance of this CT heptapeptide to CYP3A4 ERAD, CYP3A4 degradation after CT heptapeptide-deletion (CYP3A4ΔCT) was similarly examined in yeast. These findings revealed that CYP3A4ΔCT was also degraded by Ubc7p-26S proteasomal pathway, thereby indicating that this CT heptapeptide is not critical for CYP3A4 proteasomal degradation. Thus, unlike CYP2B1, CYP3A4 harbors additional/multiple structural degrons for its recruitment into the Ub-proteasomal pathway.

Mammalian hepatic cytochromes P450 (P450s) are hemoproteins instrumental in the biotransformation of various endo- and xenobiotics. It is now becoming increasingly evident that in addition to induction via transcriptional/translational activation, exposure to many substrates can alter the hepatic P450 content through substrate-induced hemoprotein stabilization as well as irreversible functional inactivation and/or enhanced degradation. P450s are integral monotopic endoplasmic reticulum (ER) proteins with their relatively hydrophobic N terminus (≈30–35 residues) embedded in the ER-membrane bilayer and the bulk of their catalytic domain exposed to the cytosol. Despite strikingly similar tertiary structures, individual hepatic P450s not only exhibit differential physiologic turnover with highly variable protein half-lives ranging from 7 to 37 h but also use distinct proteolytic loci and cellular processes (Correia, 2003, and references therein). Thus, the longer-lived CYP2B1 and CYP2C11 (half-lives of 37 and 20 h, respectively) apparently are proteolytic substrates of the autophagic-lysosomal pathway, whereas CYP3A2 and CYP3A23 ($t_{1/2} \approx 14$ h) are turned over by the ubiquitin (Ub)-dependent 26S proteasomal pathway. On

These studies were supported by National Institutes of Health grants GM44037 and DK26506. We also acknowledge the University of California San Francisco Liver Core Center Facility (Molecular Analyses/Spectrophotometry) supported by P30-DK26743.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.105.021816.

**ABBREVIATIONS:** P450, cytochrome P450; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; gp78, Autocrine motility factor receptor, AMFR; DER, degradation in endoplasmic reticulum; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HRD, 3-hydroxy-3-methylglutaryl-CoA reductase degradation; Ub, ubiquitin; Ubc, ubiquitin-conjugating enzyme; CT, C-terminal; ΔCT, C-terminal heptapeptide deletion; OD, optical density; wt, wild-type; CHIP, C terminus of Hsc70-interacting protein; AAA, ATPase-associated activities.

the other hand, CYP2E1 exhibits biphasic turnover with a "rapid phase" ($t_{1/2} \approx 7$ h), and a "slow-phase" ($t_{1/2} \approx 37$ h) and is a substrate of both proteolytic pathways (Song et al., 1989; Tierney et al., 1992). The basis for this heterogeneity and differential proteolytic targeting of each P450 is unclear but may be determined by the primary structure and/or the presence of discrete degradation signals or "degrons" harbored in each P450 structure, or generated through various posttranslational modifications (Aguiar et al., 2005). In an effort to elucidate the basis for such intrinsic differences in P450 proteolytic degradation, we have used *Saccharomyces cerevisiae* as an experimental model. Not only does *S. cerevisiae* contain the autophagic-lysosomal and Ub-dependent proteasomal pathways that are evolutionarily well conserved in mammalian cells, but it also enables convenient mechanistic characterization of individual cellular participants in these pathways through genetic screens. Such diagnostic screens have led to the identification of genes such as *PEP4* that encodes a vacuolar master proteinase (Pep4p), responsible for the post-translational processing and functional maturation of proteases involved in vacuolar degradation, the yeast counterpart of lysosomal degradation. Similar screens have also identified genes involved in the ER-associated degradation (ERAD; an Ub-dependent proteasomal process) of several integral and luminal proteins (Hampton, 2002; Kostova and Wolf, 2003). Therefore, *UBC* (Ub-conjugation), *HRD* (3-hydroxy-3-methylglutaryl-CoA reductase degradation), and *DER* (degradation in ER) genes critical in the ERAD of polytopic ER protein Hmg2p (a yeast isoform of 3-hydroxy-3-methylglutaryl-CoA reductase), and CPY* (a misfolded carboxypeptidase mutant retained in the ER lumen) have been identified. The UBC/HRD/DER proteins (Fig. 1) include: 1) ER-associated Ub-conjugating enzymes (Ubc6p and Ubc7p); 2) Cue1p, an ER anchor for docking the soluble Ubc7p; 3) Hrd2p, a functionally essential subunit of the 26S proteasomal 19S cap; 4) Hrd1p/Hrd3p complex, an ER-associated Ub-ligase (E3); and 5) cytosolic AAA ATPase Cdc48p-Ufd1p-Hrd4p chaperone complex responsible for the recognition and ER-dislocation of polyubiquitinated ER proteins, and their subsequent 26S proteasomal targeting. Using previously validated yeast strains with defined genetic disruptions in either their PEP4 or UBC/HRD/DER machinery, we have shown that CYP2B1 and CYP2C11 turnover is largely dependent on Pep4p but not on the *UBC/HRD/DER* encoded proteins, whereas that of CYP3A4 involves the Ub-conjugating enzyme Ubc7p and Hrd2p, but not Pep4p (Murray and Correia, 2001; Murray et al., 2002; Liao et al., 2005). Thus, these findings document that the proteolytic pathway used by a given P450 in the yeast is qualitatively identical to the corresponding pathway in mammalian liver, thereby validating *S. cerevisiae* as an experimental model for characterizing mammalian P450 turnover. Furthermore, because the incorporation of the CYP3A4 C-terminal (CT) heptapeptide at the CYP2B1 C terminus was sufficient to divert its degradation from predominantly vacuolar into the proteasomal pathway, we have examined the relative importance of this CT-domain as an intrinsic structural degron for CYP3A4 ERAD by characterizing the ERAD of CYP3A4 mutant after deletion of its CT heptapeptide (CYP3A4ΔCT).

Although CYP3A4 has been established as a substrate of Ub-dependent proteasomal system because of the Ubc7p/Hrd2p-dependence of its ERAD, the other key cellular participants in this process, such as the Ub-ligase, remained to be specifically identified. Given our firm exclusion of the Hrd1p/Hrd3p Ub-ligase complex in CYP3A4 ERAD (Murray and Correia, 2001), we have examined the roles of the other two Ub-ligases involved in ER-protein ubiquitination:

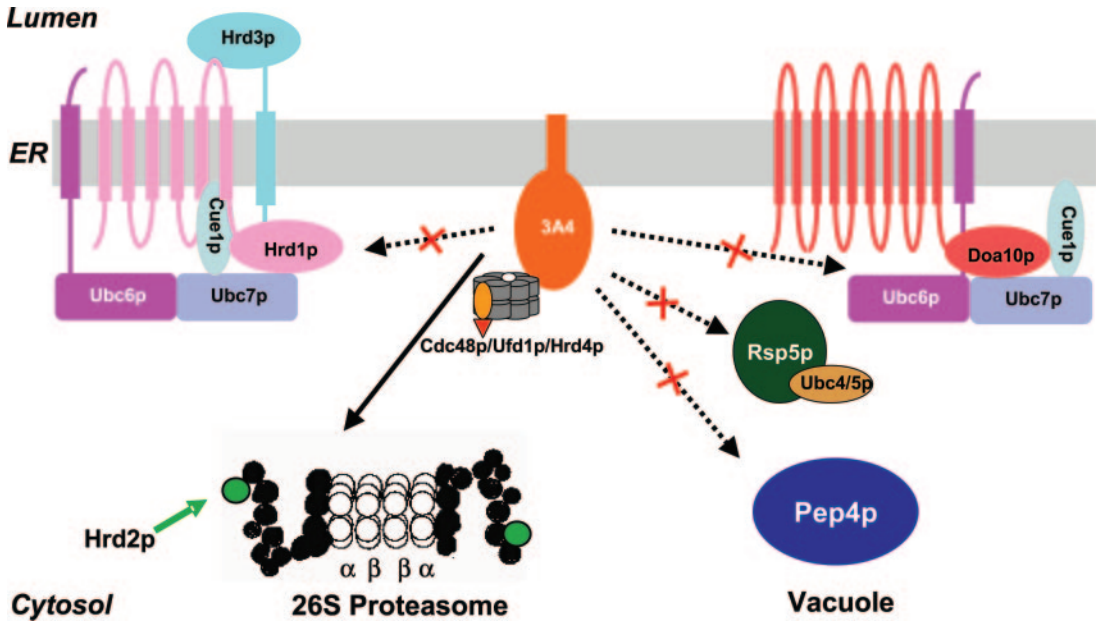


**Fig. 1.** The cellular ERAD and vacuolar proteolytic machinery of *S. cerevisiae*. The ER-anchored monotopic P450 CYP3A4 is illustrated schematically. With the exception of Ubc6p, all the other proteins (Ubc7p, Cue1p, Hrd1p/Hrd3p, Hrd2p, Cdc48p-Ufd1p-Hrd4p) are required for the *UBC/HRD*-dependent ERAD of the integral protein Hmg2p or luminal protein CPY* (data not shown) (Hampton, 2002; Kostova and Wolf, 2003). CYP3A4 ERAD in yeast is dependent on Ubc7p, Hrd2p, and Cdc48p-Ufd1p-Hrd4p complex, but not on the vacuolar *PEP4*-dependent system or any of the three known ERAD associated Ub-ligases, Hrd1p/Hrd3p, Doa10p, and Rsp5p. The solid arrow indicates the major cellular pathway of CYP3A4 ERAD. See the text for details.

Doa10p (another canonical E3) and Rsp5p. In addition, the specific involvement in CYP3A4 ERAD of Cue1p and the Cdc48p-Ufd1p-Hrd4p chaperone complex was also examined. Our findings are described below.

## Materials and Methods

### Materials

Media for yeast growth were purchased from Clontech (Mountain View, CA). Cloning reagents such as restriction enzymes, ligases and Vent polymerase were obtained from New England BioLabs (Beverly, MA). pGEM-T Easy Vector was from Promega (Madison, WI). Goat polyclonal IgGs were raised commercially against a recombinant CYP3A4 enzyme and partially purified by ammonium sulfate fractionation.

### Yeast Strains

The strains used, grouped as isogenic sets, are listed in Table 1. The methods for their construction have been described previously (Hampton et al., 1996; Wilhovsky et al., 2000; Swanson et al., 2001; Haynes et al., 2002; Huyer et al., 2004).

### Plasmids

**CYP3A4 Expression Vectors.** The rat CYP3A4 cDNA was amplified by PCR (with pGEM7-CYP3A4 encoding the full-length rat CYP3A4 as the template) and cloned into pYES2/ADH (modified from pYES2/CT, *URA*-marked, under the control of the yeast ADH1 promoter instead of the GAL1 promoter) and pYcDE-2 (*TRP*-marked, 2$\mu$ plasmid under the control of the yeast ADH1 promoter) to yield pYES2-ADH-3A4 and pYcDE-3A4, respectively.

**CYP3A4ΔCT Expression Vectors.** The expression vectors pYES2-ADH-3A4ΔCT and pYcDE-3A4ΔCT, with 21 C-terminal nucleotides deleted from the CYP3A4 coding sequence, were constructed by conventional site-directed mutagenesis techniques.

### Stationary-Chase Analyses

Yeast cell transformation was carried out according to the detailed protocol (Clontech PT3024). The conditions for the growth of the cultures have been described previously (Murray and Correia, 2001; Murray et al., 2002; Liao et al., 2005). In brief, yeast strains transformed with CYP3A4 or CYP3A4ΔCT expression vector or the corresponding empty vector were grown at either 25°C or 30°C in synthetic defined medium with appropriate supplements, as specifically indicated. Cells were harvested at an early culture stage during the logarithmic growth phase of the culture (OD of $\approx$ 0.9 at 600 nm), or at a late stage (after "stationary chase" generally 10 to 12 h after reaching an OD of 0.5 at 600 nm).

### Microsomal Preparation

Yeast microsomal fractions were prepared as described previously (Murray and Correia, 2001), except that they were enriched by removal of the other cellular contaminants by a differential sucrose gradient ultracentrifugation step exactly as described previously (Liao et al., 2005). The microsomal pellet was overlaid with potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol, 0.1 mM EDTA, and 20% (v/v) glycerol and stored at −80°C until used.

### CYP3A4/3A4ΔCT Immunoblotting Analyses

Microsomal protein (10 $\mu$g) from early- and late-stage cultures was used in these analyses. The protein content was normalized after methanol/$H_2SO_4$ precipitation and acetone washes of yeast microsomes to eliminate interference in the protein assay of variable amounts of adventitious chromophoric material. Microsomal CYP3A4/3A4ΔCT protein content was assayed by Western immunoblotting analyses and the immunoblots were densitometrically quantitated as described previously (Murray and Correia, 2001). The relative CYP3A4/3A4ΔCT content at the late stages of culture was expressed as a percentage of the corresponding CYP3A4/3A4ΔCT content (100%) at the early stage. Values depicted represent the mean ± S.D. of at the least three to five individual experiments. In addition, the phenotype of each RHY yeast strain used was confirmed by following the degradation of Myc-tagged Hmg2p in parallel by immuno slot-blotting analyses as described previously (Liao et al., 2005).

TABLE 1
Yeast strains used in these studies

| Strains | Genotype |
|---|---|
| RHY718 (*wt, HRD*) | *MATα ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2Δ trp1Δ ura3–52 +pRH244 (URA3, 6mycHMG2)* |
| RHY925 (*hrd2–1*) | *MATα ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2Δ trp1Δ URA3::6MycHMG2 hrd2–1* |
| RHY1166 (*wt, UBC*) | *MATα ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2Δ trp1Δ ura3–52::MycHMG2* |
| RHY1596 (*ubc6Δ*) | *MATα ade2–101 met2 his3Δ200 hmg2Δ4 lys2–801 hmg1::LYS2 leu2Δ trp1Δ ura3–52::MycHMG2 ubc6Δ::KanMX* |
| RHY1603 (*ubc7Δ*) | *MATα ade2–101 met2 his3Δ200 hmg2Δ4 lys2–801 hmg1::LYS2 leu2Δ trp1Δ ura3–52::MycHMG2 ubc7Δ::HIS3* |
| RHY717 (*wt, CUE1*) | *MATα ade2–101 met2 lys2–801 his3Δ200 URA3::6MYC HMG2 (Ura-6myc) hmg1Δ::LYS2 hmg2Δ::HIS3 trp1::hisG leu2Δ* |
| RHY2108 (*cue1Δ*) | *MATα ade2–101 met2 lys2–801 his3Δ200 URA3::6MYC HMG2 (Ura-6myc) hmg1Δ::LYS2 hmg2Δ::HIS3 trp1::hisG leu2Δ cue1Δ::LEU2* |
| MHY500 (*wt, DOA10*) | *MATα his3Δ200 leu2–3,112 ura3–52 lys2–801 trp1–1 gal2* |
| MHY1638 (*doa10Δ*) | *MATα his3Δ200 leu2–3,112 ura3–52 lys2–801 trp1–1 gal2 doa10Δ::HIS3* |
| MHY1702 (*doa10Δ/hrd1Δ*) | *MATα his3Δ200 leu2–3,112 ura3–52 lys2–801 trp1–1 gal2 doa10Δ::HIS3 hrd1Δ::LEU2* |
| KHY163 (*wt, RSP5*) | *MATα vph1Δ::LEU2 prc1–1 trp1–901 leu2–3,112 ura3–52 ade2–101 his3Δ200 suc2Δ9* |
| KHY355 (*rsp5–2*) | *MATα vph1Δ::LEU2 prc1–1 trp1–901 leu2–3,112 ura3–52 ade2–101 his3Δ200 suc2Δ9 rsp5–2::HIS* |
| RHY2455 (*wt, CDC48*) | *MATα ADE2 MET LYS2 HIS3 ura3–52 TRP1 leu2–3,112 HMG1 HMG2 MEV Kan(s)* |
| RHY2456 (*cdc48–2*) | *MATα ADE2 MET LYS2 HIS3 ura3–52 TRP1 LEU2 HMG1 HMG2 MEV Kan(s) cdc48–2* |
| RHY2680 (*wt, UFD1*) | *MATa ade1–100 MET LYS2 his4–519 ura3–52 TRP1 leu2–3,112 HMG1 HMG2 MEV Kan(s) ufd(+)* |
| RHY2679 (*ufd1–1*) | *MATa ade1–100 MET LYS2 his4–519 ura3–52 TRP1 leu2–3,112 HMG1 HMG2 MEV Kan(s) ufd1–1* |
| RHY697 (*wt, HRD4*) | *MATα ade2–101 met2 lys2–801 his3Δ200 ura3–52::6MYC HMG2(Ura-6myc) hmg1Δ::LYS2 hmg2Δ::HIS3 trp1::hisG leu2Δ Mev + Kan(s)* |
| RHY750 (*hrd4–1*) | *MATα ade2–101 met2 lys2–801 his3Δ200 ura3–52::6MYC HMG2(Ura-6myc) hmg1Δ::LYS2 hmg2Δ::HIS3 trp1::hisG leu2Δ Mev + Kan(s) hrd4–1* |
| RHY473 (*wt, PEP4*) | *ade2–101 met2 his3Δ200 lys2–801 ura3–52* |
| RHY106–4 (*pep4Δ*) | *ade2–101 met2 his3Δ200 lys2–801 ura3–52 pep4Δ* |

### Statistical Analyses

Analyses were performed by Student's $t$ test using Microsoft Excel (Microsoft, Redmond, WA). A value of $p < 0.05$ was considered statistically significant.

## Results

**The Relative Importance of the Cyp3A4 C Terminus to Its Ub-Proteasomal–Dependent ERAD.** For reasons discussed previously in detail (Liao et al., 2005), the degradation of CYP3A4 enzymes was routinely monitored by the "stationary chase" method because the goal was to qualitatively define the specific pathway of degradation and the roles of specific enzymes/proteins in that cellular process rather than to compare the relative rates of P450 degradation via those pathways.[1] As described previously (Murray and Correia, 2001; Liao et al., 2005), specific *UBC*, *HRD*, and *PEP4* deletion/defective mutants and corresponding isogenic wild-type (wt) *S. cerevisiae* strains were employed for such analyses. Also, as described previously, the immunodetectable levels of CYP3A4 proteins were monitored by immunoblotting analyses both at an "early" culture stage during the logarithmic growth phase when protein synthesis predominates over degradation, and at a "late" stage when protein degradation predominates over synthesis (see *Materials and Methods*). The relative loss of each protein at a "late" stage with respect to its corresponding "early" stage level thus provides an index of its degradation. When the degradation of a protein is impaired because of the disruption or lack of an essential cellular component in a specific degradation pathway, it is relatively stabilized and accumulates with time to levels higher than those seen in the corresponding wt strain. Using this approach, the relative importance of either ER-associated Ub-conjugating enzyme Ubc6p or Ubc7p in CYP3A4ΔCT ERAD was examined after expression of pYcDE-3A4ΔCT in *ubc6*Δ and *ubc7*Δ yeast strains, deficient in either enzyme. CYP3A4 was examined as a corresponding control after parallel pYcDE-3A4 expression in these yeast strains. During the stationary phase of culture, there were no statistically significant differences in CYP3A4ΔCT stabilization in the *ubc6*Δ-strain relative to that in the corresponding wt *S. cerevisiae* strain as previously observed with CYP3A4 (Murray and Correia, 2001) and now confirmed (Fig. 2). In contrast, marked stabilization of CYP3A4ΔCT was observed in the *ubc7*Δ strain, as also previously observed with CYP3A4 (Fig. 2). These findings thus indicated that CYP3A4ΔCT ERAD, like that of its parent CYP3A4 enzyme, was independent of Ubc6p but required the Ub-conjugating enzyme Ubc7p. Ubc7p involvement in ERAD of luminal and integral proteins also entails a role for Cue1p, the ER-bound anchor for Ubc7p (Biederer et al., 1997; Sommer and Wolf, 1997). Herein we show for the first time, that, as expected from their Ubc7p-requirement, the ERAD of both CYP3A4 and CYP3A4ΔCT also required Cue1p (Fig. 3). Therefore, both proteins were markedly stabilized at their "late" growth

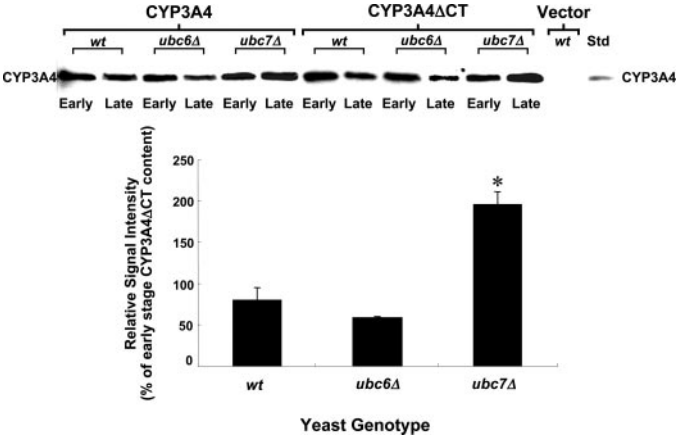


**Fig. 2.** Relative degradation of CYP3A4 or CYP3A4ΔCT in wt and *ubc*-deficient *S. cerevisiae* strains. Yeast strains transformed with the CYP3A4 or CYP3A4ΔCT expression vector (pYcDE-3A4 or pYcDE-3A4ΔCT) or the empty vector (pYcDE-2) were grown at 30°C in SD with appropriate supplements. Cells were harvested at an early culture stage during logarithmic growth phase (≈OD of 0.9 at 600 nm), or at a late stage after "stationary chase" (generally, 10 to 12 h after reaching an OD of 0.5 at 600 nm). Microsomal protein prepared from these cells was subjected to Western immunoblotting analyses with goat anti-CYP3A4 IgGs. A representative immunoblot from one of the three experiments is included. The relative densitometric quantitation of CYP3A4ΔCT immunoblots at the late stages of culture is expressed as percentage of the corresponding values (100%) at the early stage. Values represent the mean ± S.D. of at the least three individual experiments. The asterisk indicates a statistically significant difference at $p < 0.01$ between this and the corresponding wt values.

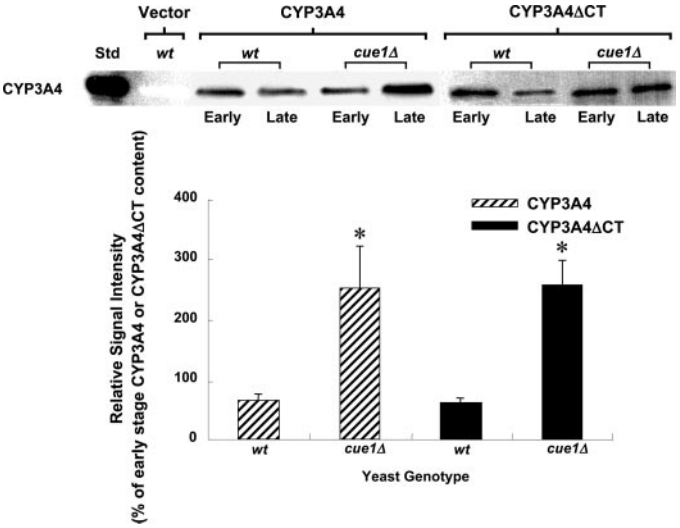


**Fig. 3.** Relative degradation of CYP3A4 or CYP3A4ΔCT in wt and *cue1*Δ *S. cerevisiae* strains. For experimental details, see Fig. 2. Values represent the mean ± S.D. of at the least three individual experiments. The asterisk indicates a statistically significant difference (at $p < 0.01$) in P450 content relative to that of the corresponding wt control strain.

[1] In yeast, unlike mammalian cells, the pulse-chase method is only viable for short-lived but not long-lived proteins such as the P450s for several reasons, discussed in detail by Kornitzer (2002). A primary reason is that, at the stationary phase of yeast growth, new protein synthesis is minimal [incorporation of the radiolabeled amino acid precursor is only ≈1.1% of that in exponentially growing yeast (Dickson and Brown, 1998)] making labeling very inefficient and variable. Another drawback of the [$^{35}$S]methionine pulse-chase is the fact that whereas most labeled protein molecules are degraded rapidly, a fraction is diverted to a stable pool (Kornitzer, 2002) and, in the case of the long-lived P450s, this fraction is large. For these reasons, we opted for the "stationary chase" method, which only enables qualitative definitions of the particular route of P450 degradation (vacuolar versus proteasomal) and the roles of certain enzymes/proteins in those pathways through the use of specific deletion/defective mutants compared with their corresponding isogenic wild type *S. cerevisiae* strains. It does not enable any absolute quantitative assessments of the relative rates of P450 degradation through these yeast proteolytic pathways. However, although we find that yeast degrade P450 proteins by the same routes as mammalian cells, the rate of P450 degradation in yeast, even if accurately determined by any suitable method, is not necessarily predictive of the rate of its degradation in mammalian cells.

stages in *cue1*Δ-strains compared with their corresponding wt yeast strains (Fig. 3).

Corresponding parallel studies of CYP3A4 and CYP3A4ΔCT in wt *HRD*- and *hrd2-1*-defective yeast strains (Fig. 4) also showed that in common with CYP3A4, CYP3A4ΔCT was stabilized in *hrd2-1* defective yeast strain but not in the wt *HRD*-strains, thereby also revealing a similar dependence of its ERAD on Hrd2p, the 19S proteasomal regulatory subunit. More importantly, these findings revealed that the CYP3A4 CT heptapeptide was not essential for either Ubc7p-dependent ubiquitination (Fig. 2) or proteasomal targeting (Fig. 4) of the CYP3A4 protein. Furthermore, similar studies in *PEP4*- and *pep4*Δ-yeast strains (Fig. 5) revealed no relative stabilization of CYP3A4ΔCT at the late stage, in common with the findings with the full-length CYP3A4 in those strains. These findings thus indicated that the CYP3A4 CT heptapeptide, albeit sufficient to divert CYP2B1 when appended to it from vacuolar into proteasomal degradation (Liao et al., 2005), was not the only "sine qua non" CYP3A4 proteasomal determinant. Apparently, CYP3A4 contains additional structural degrons that target it for Ub-dependent proteasomal degradation.

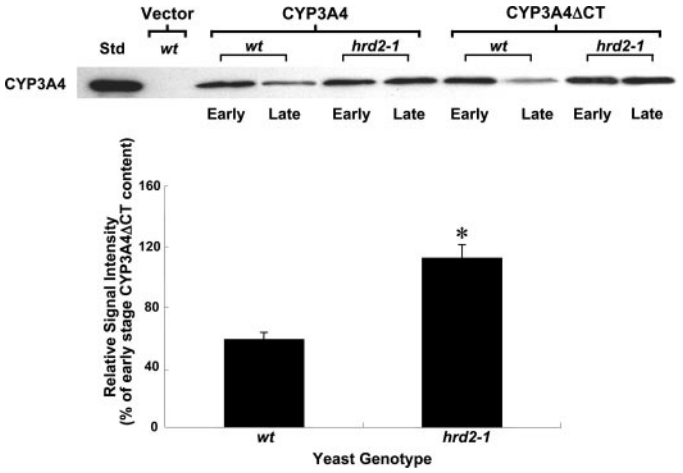


**Fig. 4.** Relative degradation of CYP3A4 or CYP3A4ΔCT in wt and *hrd2-1* mutant *S. cerevisiae* strains. For experimental details, see Fig. 2. Values represent the mean ± S.D. of at the least three individual experiments. The asterisk indicates a statistically significant ($p < 0.01$) difference in CYP3A4ΔCT content relative to that of the corresponding wt control strain.

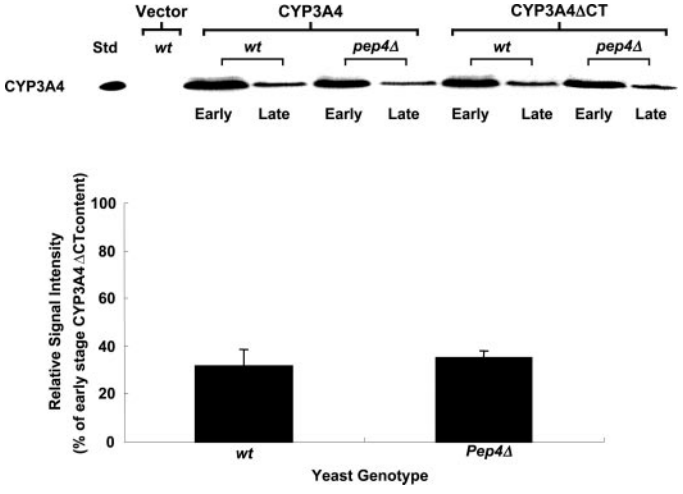


**Fig. 5.** Relative stabilization of CYP3A4 or CYP3A4ΔCT in *PEP4* and *pep4*Δ *S. cerevisiae* strains. For experimental details, see Fig. 2, except that the expression plasmids used were pYES2-ADH-CYP3A4 and pYES2-ADH-CYP3A4ΔCT. Values represent the mean ± S.D. of at the least three individual experiments with no statistically significant differences found in CYP3A4ΔCT content relative to that of the corresponding wt control strain.

**Characterization of the E3 Ub-Ligase in CYP3A4 ERAD.** The Ubc7p-dependent proteasomal targeting of most luminal and integral ER proteins also requires an E3 Ub-ligase to enable Ub-transfer from Ubc7p onto its target substrate. Because our previous studies (Murray and Correia, 2001) had conclusively excluded Hrd1p/Hrd3p, a canonical ER Ub-ligase complex shown to be involved in Hmg2p- and CPY*-ERAD, we explored the role of Doa10p, another canonical ER Ub-ligase involved in the ERAD of ER proteins and transcription factors (Johnson et al., 1998; Swanson et al., 2001; Huyer et al., 2004). Heterologous expression of CYP3A4 and CYP3A4ΔCT in wt (*DOA10*) and *doa10*Δ yeast strains followed by immunoblotting analyses at the stationary phase as described above, led to no appreciable stabilization of either protein in either strain relative to their corresponding "early stage" content (Fig. 6A). Conclusive evidence was obtained when yeast strains deficient in both Doa10p and Hrd1p were similarly tested (Fig. 6B). As seen in Fig. 6B, no relative stabilization of either CYP3A4 or CYP3A4ΔCT protein was observed in *doa10*Δ/*hrd1*Δ yeast strains, thereby convincingly excluding a role for either canonical ER-associated E3 Ub-ligase in Ubc7p-dependent CYP3A4 ERAD. Together these findings also indicated that CT-deletion had no appreciable effect on CYP3A4 ERAD. Therefore, subsequent studies were conducted with just the parental CYP3A4.

In the search for the Ub-ligase involved in Ubc7p-dependent CYP3A4 ERAD, we examined the possible participation of Rsp5p, another Ub-ligase documented to play a role in ER quality control independent of HRD/DER and DOA10 pathways (Caldwell et al., 2001; Haynes et al., 2002). Expression of CYP3A4 in wt and *rsp5-2* yeast strains resulted in no appreciable stabilization of CYP3A4 at the stationary phase relative to their early stage content

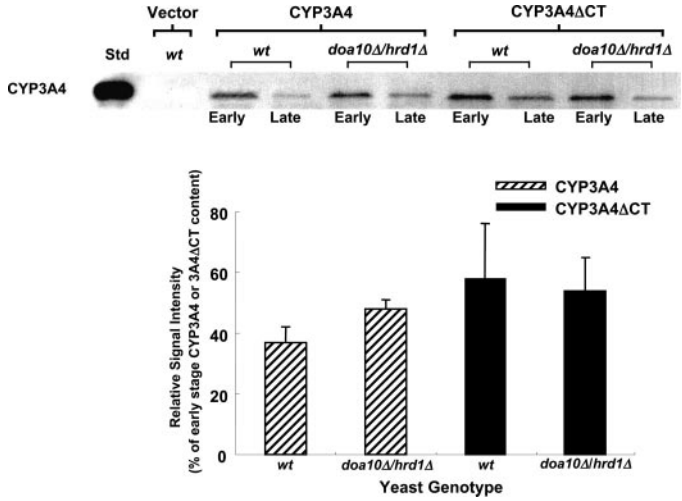


**Fig. 6.** Relative stabilization of CYP3A4 or CYP3A4ΔCT in (A) wt and *doa10*Δ *S. cerevisiae* strains and (B) wt and *doa10*Δ/*hrd1*Δ *S. cerevisiae* strains. For experimental details, see Fig. 2. Values represent the mean ± S.D. of at the least three individual experiments, with no statistically significant differences in P450 content found between the deficient and corresponding wt control strains.

(Fig. 7). These findings thus exclude the other plausible Ub-ligase in CYP3A4 ERAD.

**Role of the Cdc48p-Ufd1p-Hrd4p Complex in CYP3A4 ERAD.** Degradation of ubiquitinated integral and luminal ER-proteins requires their translocation and/or extraction from the ER-membrane into the cytosol and their delivery to the 26S proteasome, a function requiring ATP hydrolysis and generally performed by the Cdc48p-Ufd1-Hrd4p complex (Dai et al., 1998; Bays et al., 2001; Jarosch et al., 2002; Elkabetz et al., 2004; Richly et al., 2005; Ye et al., 2005). In certain instances, this complex is also believed to promote ERAD of certain target substrates by active recruitment of the relevant Ub-ligase to their ER-membrane site (Zhong et al., 2004; Ye et al., 2005). To determine whether this complex was essential in CYP3A4 ERAD, the degradation of CYP3A4 was examined in yeast strains with defects in each of the three individual components of this complex (Fig. 8). Thus, expression of CYP3A4 in *cdc48-2* yeast strains resulted in a statistically significant marked stabilization of the protein at the stationary phase relative to that in the corresponding wt yeast strain (Fig. 8). An even more striking relative CYP3A4 stabilization was observed not only in *ufd1-1* yeast strains but also in that of *hrd4-1* yeast strains, relative to their corresponding wt controls (Fig. 8). These findings thus conclusively indicated that the ERAD of the monotopic ER-anchored CYP3A4 requires the involvement of the AAA ATPase Cdc48p-Ufd1p-Hrd4p complex.

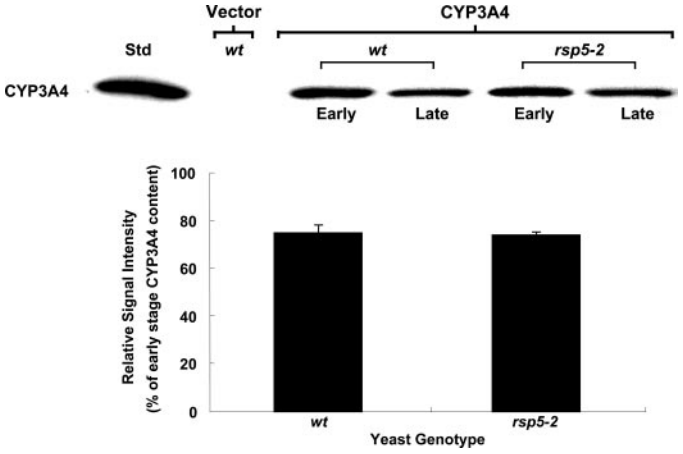


**Fig. 7.** Relative stabilization of CYP3A4 in wt and *rsp5-2* mutant *S. cerevisiae* strains. For experimental details see Fig. 2, except that the strains were grown at 25°C. Values each represent the mean ± S.D. of at the least three individual experiments, with no statistically significant differences found in CYP3A4 content between the deficient and corresponding wt control strains.

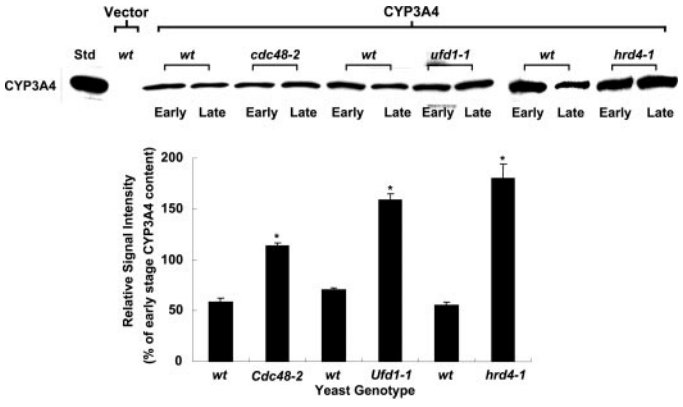


**Fig. 8.** Relative degradation of CYP3A4 in wt and *cdc48-2*-, *ufd1-1*-, and *hrd4-1*-mutant *S. cerevisiae* strains. For experimental details see Fig. 2, except that the *HRD4* and *hrd4-1* mutant strains were grown at 25°C. Values represent the mean ± S.D. of at the least three individual experiments. The asterisk indicates a statistically significant ($p < 0.01$) difference in CYP3A4 content relative to the corresponding wt control.

## Discussion

Our results indicate that CYP3A4ΔCT, like its parent CYP3A4, undergoes Ub-dependent 26S proteasomal degradation rather than vacuolar (lysosomal) degradation in *S. cerevisiae*. Such degradation of CYP3A4ΔCT also requires the cytosolic Ubc7p, but not the ER-integral Ubc6p. We now document that this Ubc7p-dependent degradation of both proteins, as expected, also requires Cue1p, the ER-membrane anchor, for recruiting the cytosolic Ubc7p to the ER-bound CYP3A4 (Fig. 3). Together, our findings indicate that deletion of the CT heptapeptide had no appreciable effect on CYP3A4 ERAD, because it affected neither its ubiquitination by Ubc7p (Fig. 2) nor its Hrd2p-dependent proteasomal degradation (Fig. 4). Moreover, such deletion also failed to increase the relative propensity of CYP3A4ΔCT for Pep4p-dependent vacuolar degradation (Fig. 5). These findings with CYP3A4 contrast our previous observations that appendage of this CT heptapeptide onto the C terminus of CYP2B1 was sufficient to divert it from vacuolar to proteasomal degradation, thereby revealing the ability of this CYP3A4 CT heptapeptide to either act as a CYP2B1 proteasomal degron or override its intrinsic vacuolar degrons (Liao et al., 2005). The lack of any significant effect on CYP3A4 ERAD by deletion of this CT heptapeptide[2] not only excludes its harboring a critical proteasomal degron but also suggests the existence of additional and/or multiple CYP3A4 degrons (possibly distributed throughout its structure) that commit it to Ub-dependent 26S proteasomal degradation. It is interesting to note in this context that multiple distributed degrons rather than discrete ones also exist along the length of the polytopic yeast Hmg2p isoform, and deletion or mutation of any single Hmg2p degron has little effect on Hmg2p ERAD (Gardner and Hampton, 1999; Doolman et al., 2004).

Although our findings above and to date (Murray and Correia, 2001; Correia et al., 2005; Liao et al., 2005) conclusively implicate the ER-associated Ubc7p/Cue1p in CYP3A4 ERAD, the Ub-ligase involved in CYP3A4 ubiquitination, if any, remains elusive. We have previously excluded a role for the integral ER Hrd1p/Hrd3p complex involved in the ERAD of many luminal and other integral ER-proteins (Hampton, 2002; Kostova and Wolf, 2003; Ahner and Brodsky, 2004; Hirsch et al., 2004; Romisch, 2005), in this process (Murray and Correia, 2001). Our findings above also exclude the two other plausible ERAD associated Ub-ligases, Doa10p and Rsp5p (Caldwell et al., 2001; Haynes et al., 2002; Huyer et al., 2004). A role for the integral ER-protein Doa10p in CYP3A4 ERAD seemed plausible given that CYP3A4 not only contains the consensus sequence PPXY ($P_{344}PTY_{347}$) apparently required to bind the WW domain of Doa10p (Swanson et al., 2001), but also the CYP3A4 crystal structure reveals that this region is apparently exposed to the cytosol

[2] It is noteworthy that the CYP3A4 crystal structure reveals that this CT heptapeptide is also entirely dispensable for CYP3A4 folding (Yano et al., 2004).

and in close proximity to CYP3A4 C terminus (Yano et al.; 2004; Fig. 9). However, no CYP3A4 stabilization was detected in yeast strains deficient in Doa10p (*doa10*Δ) or even in a yeast strain deficient in both Doa10p and Hrd1p/Hrd3p (*doa10*Δ/*hrd1*Δ), thereby revealing the independence of CYP3A4 ERAD on both these canonical ERAD Ub-ligases. These findings led us to consider the cytosolic protein Rsp5p, another Ub-ligase involved in the ERAD of certain ER-proteins (Caldwell et al., 2001; Haynes et al., 2002) as yet another plausible E3 candidate in CYP3A4 ERAD. Although Rsp5p is usually known to function in partnership with Ubc4p and Ubc5p (Gitan and Eide, 2000), reports of its Ubc7p functional association exist (Arnason et al., 2005). Furthermore, inspection of the CYP3A4 crystal structure (Yano et al., 2004) indicated that CYP3A4 also contains a cytosolically exposed $P_{405}KY_{407}$ domain, which represents a consensus sequence for Rsp5p binding/recognition (Shcherbik et al., 2004), with a potentially ubiquitinatable $K_{66}K_{67}$ cluster in strategically close spatial vicinity of this binding sequence (Fig. 9), and thus a conceivable target (Sokolik and Cohen, 1992). Similar stationary-chase analyses of CYP3A4 in *rsp5-1* and corresponding wt yeast strains indicated no role for Rsp5p in CYP3A4 ERAD (Fig. 7). Given that none of the three previously characterized ERAD associated yeast E3s are involved in CYP3A4 ERAD, the identity of the specific Ub-ligase remains presently unknown. Two possible E3 candidates in mammalian liver include the ER-anchored glycoprotein gp78/AMFR and CHIP, a cytosolic protein. gp78 is apparently related to *HRD1*, the mammalian homolog of Hrd1p (Doolman et al., 2004; Kikkert et al., 2004), which as discussed above, we have convincingly excluded in CYP3A4 ERAD in yeast (Fig. 6B; Murray and Correia, 2001). Although the possibility exists that in mammalian hepatocytes, CHIP could be responsible for ubiquitinating CYP3A as recently reported with CYP2E1 and CYP2B4 (Morishima et al., 2005), BLAST analyses yielded no homolog of CHIP in *S. cerevisiae*.[3] Thus the yeast Ub-ligase involved in CYP3A4 ERAD may be a novel protein or a known protein with a novel function, whose identity remains to be elucidated.

Our findings discussed herein also reveal a definite functional role for the ternary Cdc48p-Ufd1p-Hrd4p complex in CYP3A4 ERAD (Fig. 8). Cdc48p (and its mammalian homolog VCP/p97) is an abundant cytosolic AAA ATPase that is functionally responsible for the ATP hydrolysis required to extract substrates out of/across the ER-membrane (Dai et al., 1998; Bays et al., 2001; Jarosch et al., 2002; Elkabetz et al., 2004; Richly et al., 2005; Ye et al., 2005). On the other hand, its cofactors Ufd1p and Hrd4p are documented to bind polyUb chains and thus to recruit polyubiquitinated target substrates to the Cdc48p-complex (Bays et al., 2001; Richly et al., 2005; Ye et al., 2005). In recent years, a role for this complex in the retrotranslocation of ER-luminal proteins and extraction of integral ER proteins has been well characterized and proposed to involve both retrotranslocation/extraction of the protein across the ER-membrane into the cytosol (Dai et al., 1998; Bays et al., 2001; Jarosch et al., 2002; Elkabetz et al., 2004; Richly et al., 2005; Ye et al., 2005). Although this role for this Cdc48p complex in the ERAD of luminal and polytopic membrane proteins makes ample sense, it is unclear why the ERAD of proteins such as the P450s would require its functional participation. As discussed, P450s, although tethered to the ER-membrane via their N-terminal signal anchor, have the bulk of their catalytic domain exposed to the cytosol and thus eminently accessible to the cytosolic ubiquitination and/or 26S proteasomal degradation machinery. Thus, could the Cdc48p complex be functionally involved in the extraction of the ubiquitinated CYP3A4 out of the ER membrane and chaperoning it to the 26S proteasome? Or, as recently proposed (Zhong et al., 2004; Ye et al., 2005), could it be engaged in the active recruitment of a cytosolic Ub-ligase to the site of the ER-anchored CYP3A4 to enable its proteasomal degradation? Given the well characterized role of the Cdc48p partners Ufd1p and Hrd4p in poly-Ub chain recognition of ubiquitinated substrates, and their active involvement in CYP3A4 ERAD (Fig. 8), it is tempting to propose that a key role of this complex is to extract the ubiquitinated CYP3A4 out of the ER-membrane and shuttle it to its 26S proteasomal destruction. Indeed, consistent with this proposal, we have previously documented ER-to-cytosol translocation of polyubiquitinated, suicidally inactivated CYP3A during the course of their 26S proteasomal degradation in freshly isolated rat hepatocytes (Wang et al., 1999). In addition, we have also recently found an enhanced association of p97, the mammalian homolog of Cdc48p, with both polyubiquitinated native and structurally inactivated CYP3A2/CYP3A23 in cultured rat hepatocytes (Faouzi et al., 2005). These findings are entirely consistent with a dual role for the Cdc48p-Ufd1p-Hrd4p complex in the

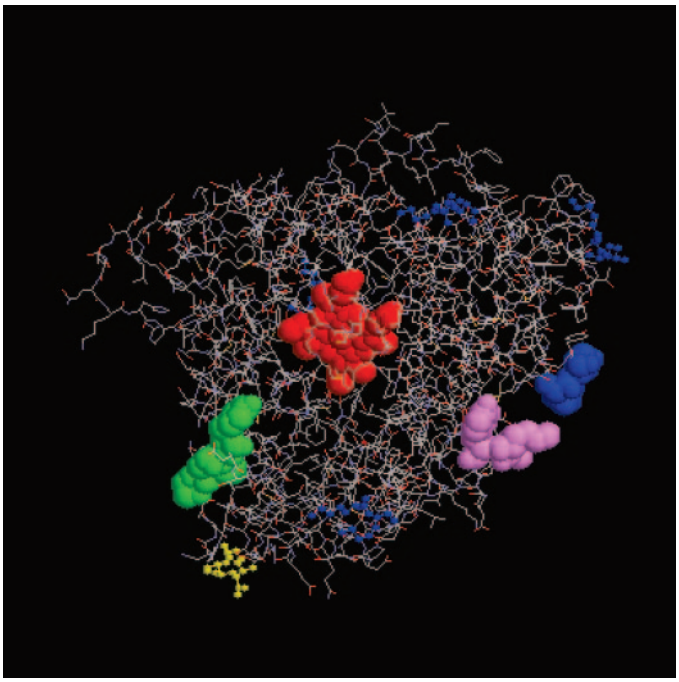

**Fig. 9.** CYP3A4: the putative Doa10p- and Rsp5p-consensus binding sequences as depicted by RasMol analyses. CYP3A4 coordinates used for its RasMol depiction were those reported by Yano et al. (2004). The prosthetic heme is shown in red. The $P_{344}PXY_{347}$, a consensus DOA10p-binding sequence (green) and $P_{405}KY_{407}$, a consensus Rsp5p-binding sequence (violet) are also shown. The $K_{66}K_{67}$ cluster is space filled in blue, and each of the other four CYP3A4 KK-clusters is also displayed as a ball-and-stick in blue. Three residues ($D_{497}GT_{499}$) of the deleted CYP3A4 CT heptapeptide are displayed in yellow.

[3] These conclusions were also confirmed via separate personal communications with Profs. R. Hampton (University of the California, San Diego) and M. Hochstrasser (Yale University).

recruitment of a Ub-ligase followed by shuttling of the ubiquitinated CYP3A4 to its proteasomal destruction. It is noteworthy that these strikingly similar cellular characteristics of CYP3A ERAD via Ub-dependent 26S proteasomal degradation in yeast and mammalian liver further validate the yeast model for the study of P450 degradation.

In summary, our findings further characterize CYP3A4 ERAD in *S. cerevisiae* by identifying additional key active cellular participants such as Cue1p and Cdc48p-Ufd1p-Hrd4p, and document the exclusion of the three most eligible ERAD-associated Ub-ligases in this process, thereby revealing the existence of another yet to be identified ERAD associated Ub-ligase in yeast. Studies are in progress to identify the elusive Ub-ligase involved in CYP3A4 ERAD in yeast and to define the precise role of the Cdc48p-Ufd1p-Hrd4p complex in this process. We have also determined that the C terminus of CYP3A4, while sufficient for diverting CYP2B1 into the Ub-dependent 26S proteasomal degradation, is not essential for committing CYP3A4 to this proteolytic pathway. Thus additional CYP3A4 structural degrons possibly distributed over its entire sequence may exist and studies are currently also in progress to identify them.

**Acknowledgments**

We gratefully thank Prof. Randy Hampton (University of California San Diego, San Diego, CA), Prof. Mark Hochstrasser and Dr. Stefan Kreft (Yale University), and Prof. Antony Cooper (University of Missouri-Kansas City) for their invaluably generous gifts of the yeast strains used in these studies as well as their helpful comments. We thank Sabrina Noel for constructing one of the CYP3A4 plasmids.

**References**

Aguiar M, Masse R, and Gibbs BF (2005) Regulation of cytochrome P450 by post-translational modification. *Drug Metab Rev* **37:**379–404.

Ahner A and Brodsky JL (2004) Checkpoints in ER-associated degradation: excuse me, which way to the proteasome? *Trends Cell Biol* **14:**474–478.

Arnason TG, Pisclevich MG, Dash MD, Davies GF, and Harkness TA (2005) Novel interaction between Apc5p and Rsp5p in an intracellular signaling pathway in *Saccharomyces cerevisiae. Eukaryot Cell* **4:**134–146.

Bays NW, Wilhovsky SK, Goradia A, Hodgkiss-Harlow K, and Hampton RY (2001) HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. *Mol Biol Cell* **12:**4114–4128.

Biederer T, Volkwein C, and Sommer T (1997) Role of Cue1p in ubiquitination and degradation at the ER surface. *Science (Wash DC)* **278:**1806–1809.

Caldwell SR, Hill KJ, and Cooper AA (2001) Degradation of endoplasmic reticulum (ER) quality control substrates requires transport between the ER and Golgi. *J Biol Chem* **276:**23296–23303.

Correia MA (2003) Hepatic cytochrome P450 degradation: mechanistic diversity of the cellular sanitation brigade. *Drug Metab Rev* **35:**107–143.

Correia MA, Sadeghi S, and Mundo-Paredes E (2005) Cytochrome P450 ubiquitination: branding for the proteolytic slaughter? *Annu Rev Pharmacol Toxicol* **45:**439–464.

Dai RM, Chen E, Longo DL, Gorbea CM, and Li CC (1998) Involvement of valosin-containing protein, an ATPase co-purified with IκBα and 26 S proteasome, in ubiquitin-proteasome-mediated degradation of IκBα. *J Biol Chem* **273:**3562–3573.

Dickson LM and Brown AJ (1998) mRNA translation in yeast during entry into stationary phase. *Mol Gen Genet* **259:**282–293.

Doolman R, Leichner GS, Avner R, and Roitelman J (2004) Ubiquitin is conjugated by membrane ubiquitin ligase to three sites, including the N terminus, in transmembrane region of mammalian 3-hydroxy-3-methylglutaryl coenzyme A reductase: implications for sterol-regulated enzyme degradation. *J Biol Chem* **279:**38184–38193.

Elkabetz Y, Shapiro I, Rabinovich E, and Bar-Nun S (2004) Distinct steps in dislocation of luminal endoplasmic reticulum-associated degradation substrates: roles of endoplasmic reticulum-bound p97/Cdc48p and proteasome. *J Biol Chem* **279:**3980–3989.

Faouzi S, Hefner C, Maher J, Medzihradszky KF, and Correia MA (2005) Characterization of the physiological turnover of native cytochrome P450 3A in cultured rat hepatocytes: a role for the cytosolic AAA ATPase p97? *Hepatology* **42**:505A.

Gardner RG and Hampton RY (1999) A 'distributed degron' allows regulated entry into the ER degradation pathway. *EMBO (Eur Mol Biol Organ) J* **18:**5994–6004.

Gitan RS and Eide DJ (2000) Zinc-regulated ubiquitin conjugation signals endocytosis of the yeast ZRT1 zinc transporter. *Biochem J* **346:**329–336.

Hampton RY (2002) Proteolysis and sterol regulation. *Annu Rev Cell Dev Biol* **18:**345–378.

Hampton RY, Gardner RG, and Rine J (1996) Role of 26S proteasome and *HRD* genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol Biol Cell* **7:**2029–2044.

Haynes CM, Caldwell S, and Cooper AA (2002) An HRD/DER-independent ER quality control mechanism involves Rsp5p-dependent ubiquitination and ER-Golgi transport. *J Cell Biol* **158:**91–101.

Hirsch C, Misaghi S, Blom D, Pacold ME, and Ploegh HL (2004) Yeast N-glycanase distinguishes between native and non-native glycoproteins. *EMBO (Eur Mol Biol Organ) Rep* **5:**201–206.

Huyer G, Piluek WF, Fansler Z, Kreft SG, Hochstrasser M, Brodsky JL, and Michaelis S (2004) Distinct machinery is required in *Saccharomyces cerevisiae* for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein. *J Biol Chem* **279:**38369–38378.

Jarosch E, Taxis C, Volkwein C, Bordallo J, Finley D, Wolf DH, and Sommer T (2002) Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nat Cell Biol* **4:**134–139.

Johnson P, Swanson R, Rakhilina L, and Hochstrasser M (1998) Degradation signal masking by heterodimerization of MATalpha2 and MATa1 blocks their mutual destruction by the ubiquitin-proteasome pathway. *Cell* **94:**217–227.

Kikkert M, Doolman R, Dai M, Avner R, Hassink G, van Voorden S, Thanedar S, Roitelman J, Chau V, and Wiertz E (2004) Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. *J Biol Chem* **279:**3525–3534.

Kornitzer D (2002) Monitoring protein degradation *Methods Enzymol* **351:**639–647.

Kostova Z and Wolf DH (2003) For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin-proteasome connection. *EMBO (Eur Mol Biol Organ) J* **22:**2309–2317.

Liao M, Zgoda VG, Murray BP, and Correia MA (2005) Vacuolar degradation of rat liver CYP2B1 in *Saccharomyces cerevisiae*: further validation of the yeast model and structural implications for the degradation of mammalian endoplasmic reticulum P450 proteins. *Mol Pharmacol* **67:**1460–1469.

Morishima Y, Peng H, Lin H, Hollenberg PF, Sunahara RK, Osawa Y, and Pratt WB (2005) Regulation of cytochrome P450 2E1 by heat shock protein 90-dependent stabilization and CHIP-dependent proteasomal degradation. *Biochemistry* **44:**16333–16340.

Murray BP and Correia MA (2001) Ubiquitin-dependent 26 S proteasomal pathway: a role in the degradation of the native human liver CYP3A4 expressed in *Saccharomyces cerevisiae*? *Arch Biochem Biophys* **393:**106–116.

Murray BP, Zgoda VG, and Correia MA (2002) Native CYP2C11: Heterologous expression in *Saccharomyces cerevisiae* reveals a role for vacuolar proteases rather than the ubiquitin-26S proteasome system in the degradation of this endoplasmic reticulum enzyme. *Mol Pharmacol* **61:**1146–1153.

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

Romisch K (2005) Endoplasmic reticulum-associated degradation. *Annu Rev Cell Dev Biol* **21:**435–456.

Shcherbik N, Kee Y, Lyon N, Huibregtse JM, and Haines DS (2004) A single PXY motif located within the carboxyl terminus of Spt23p and Mga2p mediates a physical and functional interaction with ubiquitin ligase Rsp5p. *J Biol Chem* **279:**53892–53898.

Sokolik CW and Cohen RE (1992) Ubiquitin conjugation to cytochromes *c.* Structure of the yeast ISO-1 conjugate and possible recognition determinants. *J Biol Chem* **267:**1067–1071.

Sommer T and Wolf DH (1997) Endoplasmic reticulum degradation: reverse protein flow of no return. *FASEB J* **11:**1227–1233.

Song BJ, Veech RL, Park SS, Gelboin HV, and Gonzalez FJ (1989) Induction of rat hepatic N-nitrosodimethylamine demethylase by acetone is due to protein stabilization. *J Biol Chem* **264:**3568–3572.

Swanson R, Locher M, and Hochstrasser M (2001) A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. *Genes Dev* **15:**2660–2674.

Tierney DJ, Haas AL, and Koop DR (1992) Degradation of cytochrome P450 2E1: Selective loss after labilization of the enzyme. *Arch Biochem Biophys* **29:**9–16.

Wang HF, Figueiredo Pereira ME, and Correia MA (1999) Cytochrome P450 3A degradation in isolated rat hepatocytes: 26S proteasome inhibitors as probes. *Arch Biochem Biophys* **365:**45–53.

Wilhovsky S, Gardner R, and Hampton R (2000) HRD gene dependence of endoplasmic reticulum-associated degradation. *Mol Biol Cell* **11:**1697–1708.

Yano JK, Wester MR, Schoch GA, Griffin KJ, Stout CD, and Johnson EF (2004) The structure of human microsomal cytochrome P450 3A4 determined by X-ray crystallography to 2.05A resolution. *J Biol Chem* **279:**38091–38104.

Ye Y, Shibata Y, Kikkert M, van Voorden S, Wiertz E, and Rapoport TA (2005) Inaugural Article: recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. *Proc Natl Acad Sci USA* **102:**14132–14138.

Zhong X, Shen Y, Ballar P, Apostolou A, Agami R, and Fang S (2004) AAA ATPase p97/valosin-containing protein interacts with gp78, a ubiquitin ligase for endoplasmic reticulum-associated degradation. *J Biol Chem* **279:**45676–45684.

**Address correspondence to:** M. A. Correia, Dept. of Cellular and Molecular Pharmacology, Box 2280, University of California, San Francisco, CA 94143-2280. E-mail: mariac@itsa.ucsf.edu