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

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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 (CYP3A4/CT) was similarly examined in yeast. These findings revealed that CYP3A4/ΔCT 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 (halflives of 37 and 20 h, respectively) apparently are proteolytic substrates of the autophagic-lysosomal pathway, whereas CYP3A2 and CYP3A23 (t1/2 ≈ 14 h) are turned over by the ubiquitin (Ub)-dependent 26S proteasomal pathway. On

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.

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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 posttranslational 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-conjugating), *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:
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 pGEM-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, 2μ 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 ~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 μg) from early- and late-stage cultures was used in these analyses. The protein content was normalized after methanol/H2SO4 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 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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHY718 (wt, HRD)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2a trpl1 ura3–52 pRH244 (URA3, 6mycHMG2)</td>
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<tr>
<td>RHY925 (hrd2–1)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2a trpl1 ura3–52 URA3::6mycHMG2 hrd2–1</td>
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<tr>
<td>RHY1166 (wt, UBC)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2a trpl1 ura3–52::MycHMG2</td>
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<tr>
<td>RHY1596 (ubc6Δ)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2a trpl1 ura3–52::MycHMG2 ubc6Δ::KanMX</td>
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<tr>
<td>RHY1603 (ubc7Δ)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2a trpl1 ura3–52::MycHMG2 ubc7Δ::HIS3</td>
</tr>
<tr>
<td>RHY717 (wt, CUE1)</td>
<td>MATa ade2–101 met2 lys2–801 his3Δ200 URA3::6MYC HMG2 (Ura-6myc) hmg1::LYS2 leu2a::HIS3 trpl1::HisG leu2a</td>
</tr>
<tr>
<td>RHY2108 (cue1Δ)</td>
<td>MATa ade2–101 met2 lys2–801 his3Δ200 URA3::6MYC HMG2 (Ura-6myc) hmg1::LYS2 leu2a::HIS3 trpl1::HisG leu2a cue1Δ::LEU2</td>
</tr>
<tr>
<td>RHY500 (wt, DOA10)</td>
<td>MATa his3Δ200 leu2–3,112 ura3–52 lyr2–801 trpl1–1 gal2</td>
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<tr>
<td>RHY1638 (doa10Δ)</td>
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<td>RHY1702 (doa10Δ/hrd1Δ)</td>
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<tr>
<td>RHY163 (wt, RSP5)</td>
<td>MATa uph1Δ::LEU2 pre-1 trp1–1 ura3–52 ade2–101 his3Δ200 suc239</td>
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<tr>
<td>RHY355 (rsp5–2)</td>
<td>MATa uph1Δ::LEU2 pre-1 trp1–1 ura3–52 ade2–101 his3Δ200 suc239 rps5–2::HIS3</td>
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<td>RHY2455 (wt, CDC48)</td>
<td>MATa ade2 MET4 LIFE HMG2 ura3–52 TRP1 leu2a::HIS3 hmg1::LYS2 leu2a::MycHMG2 MEV Kan(s)</td>
</tr>
<tr>
<td>RHY9456 (cdc48–2)</td>
<td>MATa ade2 MET4 LIFE HMG2 ura3–52 TRP1 leu2a::HIS3 hmg1::LYS2 leu2a::MycHMG2 MEV Kan(s) cdc48–2</td>
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<td>RHY2890 (wt, UFD1)</td>
<td>MATa ade2–100 MET4 LIFE HMG2 ura3–52 TRP1 leu2a–3,115 HMG1 HMG2 MEV Kan(s) ufd1–1</td>
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<td>RHY2679 (ufdl–1)</td>
<td>MATa ade2–100 MET4 LIFE HMG2 ura3–52 TRP1 leu2a–3,115 HMG1 HMG2 MEV Kan(s) ufd1–1</td>
</tr>
<tr>
<td>RHY697 (wt, HRD4)</td>
<td>MATa ade2–101 met2 lys2–801 his3Δ200 ura3–52::6MYC HMG2 (Ura-6myc) hmg1::LYS2 leu2a::HIS3 trpl1::HisG leu2a::MycHMG2 MEV Kan(s)</td>
</tr>
<tr>
<td>RHY750 (hrd4–1)</td>
<td>MATa ade2–101 met2 lys2–801 his3Δ200 ura3–52::6MYC HMG2 (Ura-6myc) hmg1::LYS2 leu2a::HIS3 trpl1::HisG leu2a::MycHMG2 MEV Kan(s) hrd4–1</td>
</tr>
<tr>
<td>RHY473 (wt, PEP4)</td>
<td>ade2–101 met2 his3Δ200 lys2–801 ura3–52</td>
</tr>
<tr>
<td>RHY106–4 (pep4Δ)</td>
<td>ade2–101 met2 his3Δ200 lys2–801 ura3–52 pep4Δ</td>
</tr>
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Degradation of CYP3A4 and CYP3A4ΔCT in S. cerevisiae

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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-conjuring enzyme Ubc6p or Ubc7p in CYP3A4ACT ERAD was examined after expression of pYcDE-3A4CT in ubc6Δ and ubc7Δ 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 ubc6Δ 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 ubc7Δ 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-conjuring 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-requrement, the ERAD of both CYP3A4 and CYP3A4ΔCT also required Cue1p (Fig. 3). Therefore, both proteins were markedly stabilized at their “late” growth phase relative to the corresponding control values (Fig. 2).

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 and incorporation of the radiolabeled amino acid precursor is only ~1% of that in exponentially growing yeast (Dickson and Brown, 1998) making labeling very inefficient and variable. Another drawback of the [35S]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 higher than those seen in the corresponding wt strain. During the stationary phase of culture, there were no statistically significant differences in CYP3A4ΔCT stabilization in the ubc6Δ 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 ubc7Δ 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-conjuring 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-requrement, the ERAD of both CYP3A4 and CYP3A4ΔCT also required Cue1p (Fig. 3). Therefore, both proteins were markedly stabilized at their “late” growth phase relative to the corresponding control values (Fig. 2).

**Fig. 2.** Relative degradation of CYP3A4 or CYP3A4ACT in wt and ubc6-deficient S. cerevisiae strains. Yeast strains transformed with the CYP3A4 or CYP3A4ACT expression vector (pYcDE-3A4 or pYcDE-3A4ACT) 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 CYP3A4ACT 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 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 CYP3A4ACT in wt and cue1Δ S. cerevisiae strains. For experimental details, see Fig. 2. Values represent the mean ± S.D. of at 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.
stages in \textit{cue1}\textDelta-strains compared with their corresponding wt yeast strains (Fig. 3).

Corresponding parallel studies of CYP3A4 and CYP3A4\textDelta CT in wt HRD- and \textit{hrd2}\textDelta-1-defective yeast strains (Fig. 4) also showed that in common with CYP3A4, CYP3A4\textDelta CT was stabilized in \textit{hrd2}\textDelta-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 \textit{pep4}\Delta yeast strains (Fig. 5) revealed no relative stabilization of CYP3A4\textDelta CT 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.

\textbf{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 CYP3A4\textDelta CT in wt (\textit{DOA10}) and \textit{doa10}\Delta 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 CYP3A4\textDelta CT protein was observed in \textit{doa10}\Delta/\textit{hrd1}\Delta 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 \textit{rps5-2} yeast strains resulted in no appreciable stabilization of CYP3A4 at the stationary phase relative to their early stage content.
strains were grown at 25°C. Values each represent the mean ± S.D. of at least three individual experiments, with no statistically significant differences found in CYP3A4 content relative to the corresponding wt control strains. For experimental details see Fig. 2, except that the corresponding wt control strains.

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-Ufd1p-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.

Figure 7. Relative stabilization of CYP3A4 in wt and rps5-2 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 least three individual experiments, with no statistically significant differences found in CYP3A4 content between the deficient and corresponding wt control strains.

Figure 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 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 CYP3A4ACT, like its parent CYP3A4, undergoes Ub-dependent 26S proteasomal degradation rather than vacuolar (lysosomal) degradation in S. cerevisiae. Such degradation of CYP3A4ACT 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. 4). 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 CYP3A4ACT for Psp4p-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 does not only exclude 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, Dooa10p and Rsp5p (Caldwell et al., 2001; Haynes et al., 2002; Huyer et al., 2004). A role for the integral ER-protein Dooa10p in CYP3A4 ERAD seemed plausible given that CYP3A4 not only contains the consensuse sequence PPXY (P344PTY347) but also 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 (doa10Δ) or even in a yeast strain deficient in both Doa10p and Hrd1p/Hrd3p (doa10Δ/hrd1Δ/hrd3Δ), 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 P405KY407 domain, which represents a consensus sequence for Rsp5p binding/recognition (Scherbik et al., 2004), with a potentially ubiquitinating K406K407 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 rsp5Δ-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. 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 shunt 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

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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 shunting 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 our findings, our further characterize CYP3A4 ERAD in S. cerevisiae by identifying additional key active cellular participants such as Cue1p and Cdc48p-Ufl1p-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-Ufl1p-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.

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