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

Mingxiang Liao, Victor G. Zgoda, Bernard P. Murray, and Maria Almira Correia

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

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ABSTRACT

Mammalian hepatic cytochromes P450 (P450s) are endoplasmic reticulum (ER)-anchored hemoproteins with highly variable half-lives. CYP3A4, the dominant human liver drug-metabolizing enzyme, and its rat liver orthologs undergo ubiquitin (Ub)-dependent 26S proteasomal degradation after suicide inactivation or after heterologous expression in Saccharomyces cerevisiae. In contrast, rat liver CYP2C11 is degraded by the vacuolar "lysosomal" pathway when similarly expressed in yeast. The structural determinants that commit P450s to proteasomal or lysosomal degradation are unknown. To further validate S. cerevisiae as a model for exploring mammalian P450 turnover, the degradation of phenobarbital-inducible liver CYP2B1, an enzyme reportedly degraded via the rat hepatic autophagic-lysosomal pathway, was examined in a yeast strain (pep4Δ) deficient in vacuolar proteolysis. Although CYP2B1 was equivalently expressed in both strains during early logarithmic growth, its degradation was retarded in pep4Δ strain, remaining at a level 5-fold higher than that in PEP4 yeast when monitored at the stationary phase. No comparable CYP2B1 stabilization was detected in yeast genetically deficient in the ER Ub-conjugating enzyme Ubc6p or Ubc7p or defective in 19S proteasomal subunit Hrd2p. Thus, as in the rat liver, CYP2B1 is a target of vacuolar/lysosomal rather than proteasomal degradation in yeast, thereby further validating this model for mammalian P450 turnover. It is intriguing that a chimeric protein, CYP2B1-3A4CT, with the CYP3A4 C-terminal heptapeptide grafted onto the CYP2B1 C terminus, was proteasomally degraded after similar expression. Such diversion of CYP2B1 from its predominantly vacuolar degradation suggests that the CYP3A4 heptapeptide could either actively signal its proteasomal degradation or block its vacuolar proteolysis.

Mammalian liver cytochromes P450 (P450s) are monotopic, endoplasmic-reticulum (ER)-anchored proteins engaged in the biotransformation of various endo- and xenobiotics. They are a superfamily of constitutive and inducible hemoproteins whose hepatic content, diversity, and/or function can be modulated by their substrates through altered synthesis and/or turnover (Correia, 1991, 2003). Studies of individual isoforms reveal that P450 protein turnover is highly variable (t1/2 = 7–37 h), but the basis for this heterogeneity has yet to be elucidated. A major deterrent to the characterization of P450 turnover is the lack of a suitable experimental model wherein this physiological process can be faithfully reproduced. For instance, the ER-bound P450s phenobarbital (PB)-inducible CYP2B1 and ethanol/acetonecomplexed CYP2E1 exhibit protein half-lives of ~37 h and are degraded by the autophagic-lysosomal pathway in the rat liver (Masaki et al., 1987; Ronis and Ingelman-Sundberg, 1990).

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ABBREVIATIONS: P450s, cytochromes P450; ER, endoplasmic reticulum; DER, degradation in endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; HRD, 3-hydroxy-3-methylglutaryl-CoA reductase degradation; PB, phenobarbital; Ub, ubiquitin; UBC, ubiquitin conjugation; PCR, polymerase chain reaction; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DTT, dithiothreitol; E-64, N-(trans-epoxy succinyl)-L-leucine 4-guanidinobutylamide; TAO, troleandomycin; wt, wild type; OD, optical density; SD, selective dropout.
In contrast, CYP2B1 and CYP2E1 stably expressed in HeLa cells exhibit considerably shorter half-lives (t1/2 ~ 8.7 and 3.7 h, respectively) and are degraded in a ubiquitin (Ub)-independent process, blocked by specific proteasomal but not lysosomal inhibitors (Huan et al., 2004). The basis for this accelerated P450 protein turnover and altered degradation route is unclear.

In the search for a model for the mechanistic characterization of mammalian liver P450 turnover, we have used Saccharomyces cerevisiae. This model has been used to characterize the ER-associated degradation (ERAD) of several integral and luminal proteins (Hampton, 2002a,b; Kostova and Wolf, 2003), most notably the polytopic ER protein Hmg2p (the yeast form of 3-hydroxy-3-methylglutaryl-CoA reductase) (Fig. 1), and CPY* (a misfolded carboxypeptidase mutant retained in the ER lumen). Through genetic screens, UBC (Ub conjugation), HRD (3-hydroxy-3-methylglutaryl-CoA reductase degradation), and DER (degradation in ER) genes have been identified that are critical for Hmg2p and CPY* ERAD (Hampton, 2002a,b; Kostova and Wolf, 2003). This UBC/HRD/DER machinery includes the following: 1) ER-associated Ub-conjugating enzymes (Ubc1p, Ubc6p, and Ubc7p); 2) Hrd2p, a 19S subunit that is essential for 26S proteasome function; 3) Hrd1p/Hrd3p complex, an ER-associated Ub ligase; and 4) Cdc48p-Ufd1p-Hrd4p complex responsible for the recognition of polyubiquitinated ER proteins, their ER dislocation, and subsequent delivery to the proteasome function; 3) Hrd1p/Hrd3p complex, an ER-associated Ub ligase; and 4) Cdc48p-Ufd1p-Hrd4p complex responsible for the recognition of polyubiquitinated ER proteins, their ER dislocation, and subsequent delivery to the proteasome function.

These findings and the possible role of the P450 C terminus in the differential proteolytic targeting of these proteins are discussed.

Materials and Methods

Materials. Media for yeast growth were purchased from BD Biosciences Clontech (Palo Alto, 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). Rabbit polyclonal IgGs were raised commercially against CYP2B1 enzyme (purified from PB-pre-treated rat livers) and purified by Protein A-Sepharose affinity chromatography.

Yeast Strains. The strains used, grouped as isogenic sets, are listed in Table 1. The methods for their construction have been described previously (Hampton and Rine, 1994; Hampton et al., 1996; Wilhovsky et al., 2000).

Plasmids

CYP2B1 Expression Vectors. The rat CYP2B1 cDNA was amplified by PCR (with pSW1 encoding the full-length rat CYP2B1 as the template) and cloned into pYES2/CT (URA-marked, under the control of the yeast GAL1 promoter) and pYeCDE-2 (TRP-marked 2-μ plasmid under the control of the yeast ADH1 promoter) to yield pYES2-2B1 and pYeCDE-2B1, respectively. pKKCYP2B1(His)4 encoding a full-length CYP2B1 protein with a Glu2→Ala mutation for codon optimization (John et al., 1994) was a generous gift from Professor J. R. Halpert (University of Texas, Galveston, TX).

CYP2B1-3A4CT Expression Vectors. Using PCR with pYES2-2B1 as the template, the 21 C-terminal nucleotides from the CYP3A4 cDNA to generate CYP2B1-3A4CT. The CYP2B1-3A4CT fusion was inserted into pYeCDE-2 and pYES2/ADH (modified from pYES2/CT, URA-marked, under the control of the yeast ADH1 promoter instead of the GAL1 promoter) to yield pYeCDE2B1-3A4CT and pYES2-ADH/2B1-3A4CT, respectively. The following primers were used for CYP2B1-3A4CT construction: PCR forward primer, 5′-CGT CCA CCC GTC GTG GTA ACA CCA GGA CCAT-3′; PCR reverse primer, 5′-ATC AGG CTC CAC TTA CGG TGC CAT CCC GAG CTG AGA AGC-3′; pKK2B1-3A4CT(His), was similarly constructed by inserting the nucleotides coding for the seven C-terminal CYP3A4 residues in-frame into the His6-tag by conventional site-directed mutagenesis techniques.
Yeast Cell Transformation. This was carried out according to the detailed protocol (BD Biosciences Clontech PT3024). The conditions for the growth of the cultures have been described previously (Murray and Correia, 2001; Murray et al., 2002). In brief, yeast strains transformed with CYP2B1 expression vector or the corresponding empty vector were grown at 30°C in SD medium with appropriate supplements. Cells were harvested at an early culture stage during the logarithmic growth phase of the culture (OD = 1.0 at 600 nm) or at a late stage (after “stationary chase”, generally 20 to 24 h after reaching an OD of 0.5 at 600 nm).

Bacterial Expression and Functional Reconstitution of CYP2B1 and CYP2B1-3A4CT. CYP2B1 and CYP2B1-3A4CT were expressed in Escherichia coli CYP2B1 and CYP2B1-3A4CT. The enzymes were partially purified from CHAPS-solubilized bacterial membranes by nickel-nitrilotriacetic acid affinity chromatography, followed by extensive dialysis of the imidazole (200 mM)-eluted P450 proteins. The enzymes were reconstituted with P450 reductase, and their testosterone probe as described previously (Wang et al., 1998). The enzymes were partially purified from CHAPS-solubilized bacterial membranes by nickel-nitrilotriacetic acid affinity chromatography, followed by extensive dialysis of the imidazole (200 mM)-eluted P450 proteins. The enzymes were reconstituted with P450 reductase, and their testosterone probe as described previously (He et al., 1996).

Microsomal Preparation. Yeast microsomal fractions were prepared as described previously (Murray and Correia, 2001), except that they were enriched by the removal of the other cellular contaminants by a differential sucrose gradient ultracentrifugation step. In brief, spheroplasts were suspended in 2 volumes of 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM MgSO₄, 0.1 mM EDTA, 10 mM potassium acetate, 1 mM DTT, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 mg/l leupeptin, 10 mg/l aprotonin, 1 μM bestatin, 1 μM E-64, and 1 mg/l pepstatin). The spheroplast suspension was sonicated and then sedimented at 15,000 g for 15 min. The 15,000 g supernatant was then carefully overlaid over 20% sucrose and centrifuged at 100,000 g for 2 h. The sucrose layer containing light microsomes and the microsomal pellet were resuspended in 15 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM DTT and 0.1 mM EDTA, resedimented at 100,000 g for 1 h, and “washed” as described previously (Murray and Correia, 2001). The microsomal pellet was overlaid with potassium phosphate buffer, pH 7.4, containing 1 mM DTT, 0.1 mM EDTA, and 20% (v/v) glycerol and stored at −80°C until used.

Fig. 1. The cellular ERAD and vacuolar proteolytic machinery of S. cerevisiae. The ER-bound polytopic Hmg2p and the monotopic P450s (2B1, 2B1-3A4CT, 3A4, and 2C11) are illustrated schematically. With the exception of Ubc6p, all of the other proteins (Ubc7p, Cue1p, Hrd1p/Hrd3p, and Hrd2p) have been shown to be required for the HRD-dependent ERAD of the integral protein Hmg2p (illustrated above) or lumenal protein CPY* (data not illustrated) (Hampton, 2002a,b; Kostova and Wolf, 2003). Ubc7p and Hrd2p are also required for CYP3A4 ERAD in yeast. The yeast vacuolar PE4-dependent system is also shown. The solid arrows indicate the major pathways for the degradation of particular P450 proteins. See the text for details.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>RHY718 (wt, HRD)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hisG ura3–52 hisG ura3–52 HMG2 ubc6 ubc7 ubc7::URA3 ade2::HIS3 pepstatin (URA3, 6mycHMG2)</td>
</tr>
<tr>
<td>RHY609 (hrd1Δ)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hisG ura3–52 hisG ura3–52 HMG2 ubc6 ubc7 ubc7::URA3 ade2::HIS3 pepstatin (URA3, 6mycHMG2)</td>
</tr>
<tr>
<td>RHY925 (hrd2Δ)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hisG ura3–52 hisG ura3–52 HMG2 ubc6 ubc7 ubc7::URA3 ade2::HIS3 pepstatin (URA3, 6mycHMG2)</td>
</tr>
<tr>
<td>RHY749 (hrd3Δ)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hisG ura3–52 hisG ura3–52 HMG2 ubc6 ubc7 ubc7::URA3 ade2::HIS3 pepstatin (URA3, 6mycHMG2)</td>
</tr>
<tr>
<td>RHY1166 (wt, UBC)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hisG ura3–52 hisG ura3–52 HMG2 ubc6 ubc7 ubc7::URA3 ade2::HIS3 pepstatin (URA3, 6mycHMG2)</td>
</tr>
<tr>
<td>RHY1586 (ube6Δ)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hisG ura3–52 hisG ura3–52 HMG2 ubc6 ubc7 trp1Δ ade2::HIS3 pepstatin (URA3, 6mycHMG2)</td>
</tr>
<tr>
<td>RHY1603 (ube7Δ)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hisG ura3–52 hisG ura3–52 HMG2 ubc6 ubc7 ade2::HIS3</td>
</tr>
<tr>
<td>RHY1604 (ube6Δ/7Δ)</td>
<td>MATa ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hisG ura3–52 hisG ura3–52 HMG2 ubc6 ubc7 ade2::HIS3</td>
</tr>
<tr>
<td>RHY473 (wt, PE4)</td>
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</tr>
<tr>
<td>RHY106-4 (pep4Δ)</td>
<td>ade2–101 met2 his3Δ200 lys2–801 ura3–52 pep4Δ</td>
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CYP2B1 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 CYP2B1 protein content was assayed by Western immunoblotting analyses similar to those described previously (Murray and Correia, 2001), except that the electroblotted membranes were exposed overnight to rabbit polyclonal anti-rat CYP2B1 IgGs (1:7000, v/v) as the primary antibody. The CYP2B1 immunoblots were densitometrically quantified as described previously (Murray and Correia, 2001; Murray et al., 2002). The relative CYP2B1 content at the late stages of culture was expressed as a percentage of the corresponding CYP2B1 content (100%) at the early stage. Values depicted represent the mean ± S.D. of at least three to five individual experiments.

Slot-Blotting Analyses. The phenotype of the yeast strain used was validated by following the degradation of Myc-tagged Hmg2p in parallel by immunoblotting analyses as described previously (Murray and Correia, 2001), except that polyclonal anti-Myc IgGs were used.

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

Results

Independence of CYP2B1 Degradation from the Normal UBC/HRD ERAD Machinery. In preliminary experiments, we confirmed that only yeast strains transformed with the CYP2B1 expression vectors, but not the empty vectors, were capable of expressing CYP2B1. The authenticity of the UBC and HRD gene functionality (or lack thereof) of these strains was also verified by examining the degradation of chromosomally integrated 6Myc-HMG2 by immunoblotting analyses, essentially as described previously (Murray and Correia, 2001; Murray et al., 2002; data not shown). Of the available protein turnover methods in yeast (Hampton et al., 1996; Kornitzer, 2002), the “stationary chase” was found to be the most suitable method for monitoring the degradation of the relatively long-lived protein CYP2B1. The specific goal was to qualitatively define the particular route of degradation and the roles of certain enzymes/proteins in that pathway through the use of specific HRD, UBC, or PEP4 deletion/defective mutants and corresponding wild-type isogenic S. cerevisiae strains rather than to quantitatively assess the relative rates of P450 degradation through these yeast proteolytic pathways. To validate the “stationary chase” method for assessing P450 degradation, CYP2B1 turnover was also monitored by the pulse-chase technique.

[35S]Methionine was added to the RHY1166 culture in a methionine/cysteine-free medium at a culture density of ∼1.0 (early stage) and chased with unlabeled methionine/cysteine after 1 h. Labeled CYP2B1 was assayed by fluorography of immunoprecipitates of yeast lysates (n = 3 separate experiments). After 21 h (∼late stage), we found that 43 ± 5% of the labeled CYP2B1 remained (i.e., loss of ∼57% of early-stage content), and this corresponded very nicely to the value determined by the “stationary chase” method (38 ± 10% remaining at 21 h, or a loss of ∼62%). However, unlike the total microsomal CYP2B1 content, which continued to decline, the radiolabeled CYP2B1 content reached a steady level at approximately this time (Kornitzer, 2002). Because the stationary chase was performed with microsomal preparations, whereas the immunoprecipitation analyses were with yeast lysates, it is possible that the immunoprecipitated CYP2B1 content at later (>21 h) times reflects both its microsomal and nonmicrosomal pools (possibly sequestered within the autophagic vacuoles).⁵

The relative importance of either ER-associated Ub-conjugating enzyme Ubc6p or Ubc7p, or both of these enzymes, in CYP2B1 degradation was examined by pYcDE-2B1 expression in ubc6Δ, ubc7Δ, and ubc6Δ/ubc7Δ yeast strains deficient in either or both enzymes. During the stationary phase of culture, there were no statistically significant differences in CYP2B1 stabilization in any of these strains relative to that in the corresponding wt S. cerevisiae strain (Fig. 2). These findings thus indicated that CYP2B1 degradation was independent of either of the two Ub-conjugating enzymes shown previously to be involved in ERAD.

Similar expression analyses in wt and hrd2Δ-1, hrd1Δ, and hrd3Δ S. cerevisiae strains revealed comparable CYP2B1 degradation and thus no CYP2B1 stabilization in any of the hrd-defective/deficient strains relative to the corresponding wt strain when monitored at the stationary phase of culture (Fig. 3). These findings indicated that CYP2B1 degradation was independent of Hrd2p, and thus of the 26S proteasome, a feature in common with the degradation of CYP2C11 but not with that of CYP3A4. Furthermore, in common with the degradation of both CYP2C11 and CYP3A4, the degradation of CYP2B1 was also independent of the Hrd1p/Hdr3p Ub-ligase complex responsible for Hmg2p ubiquitination in S. cerevisiae.

PEP4 Gene Dependence of CYP2B1 Degradation. pYES2-2B1 expression analyses revealed a marked (∼5-fold) relative CYP2B1 stabilization in the pep4Δ strain compared with the content in the wt (PEP4) strain at the stationary phase of culture (Fig. 4). This level of retardation in degradation was thus much more pronounced than the 2-fold stabilization of CYP2C11 observed previously in this same strain (Murray et al., 2002), thereby revealing the critical importance of the vacuolar pathway for CYP2B1 degradation in yeast. These findings are consistent with the lysosomal degradation of CYP2B1 documented in the rat liver and thus provide further validation of this yeast model for the characterization of mammalian P450 turnover.

CYP2B1-3A4CT Degradation in Yeast. The differential sorting of mammalian ER-anchored CYP2B1, CYP2C11, and CYP3A4 into two distinct proteolytic pathways not only underscores the mechanistic diversity of the intracellular P450 degradation process but also suggests that structural/molecular determinants may dictate the choice of degradation pathways. However, intrinsic determinants of such differential targeting have yet to be identified and could be located

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⁵ This raises the issue of intracellular “protein trafficking”, which undoubtedly must occur during P450 degradation because the ER-anchored proteins are extracted from the ER membrane and translocated either to the vacuoles or the proteasome in the cytosol or even, albeit minutely, to the cell membrane. As explained above, from the design of the experiment with qualitative pairwise comparisons of mutant strain versus isogenic wild type, we believe that our general qualitative conclusions of vacuolar versus proteasomal routes of degradation remain valid even if there are changes in trafficking. Thus, if the hrd2-1 defect (known to inactivate the proteasome) has no effect on CYP2B1 stability, whereas deletion of Pep1p (known to inactivate the vacuole, albeit indirectly, through impairment of vacuolar protease maturation) stabilizes the protein, then the logical conclusion is that the degradation of CYP2B1 is dependent on the vacuole (even if only indirectly). We believe that the same argument could apply to potential changes in protein trafficking.
anywhere on the P450 protein. The P450 N terminus is embedded in the ER membrane, and this inaccessibility makes it a priori a considerably less plausible proteolytic targeting sequence than the cytosol-exposed P450 C-terminal domain (Williams et al., 2004; Yano et al., 2004). Thus, as an initial approach to identifying structural features that commit P450s to either of these two pathways, we examined the potential role of the extreme C terminus of the protein in the differential proteolytic targeting of P450s. For this purpose, the degradation of a chimeric protein (CYP2B1-3A4CT) (Fig. 5), consisting of the full-length CYP2B1 with seven CYP3A4 C-terminal residues appended at its C terminus, was examined in PEP4 and pep4Δ yeast strains (Fig. 6). In contrast to the dramatic stabilization of unmodified CYP2B1 (Fig. 4), no corresponding stabilization of the chimeric CYP2B1-3A4CT protein was observed in the pep4Δ strain at the stationary growth stage (Fig. 6), thereby revealing that this CYP2B1 chimeric protein was no longer dependent on the vacuolar pathway for its degradation. Consistent with this apparent switch in the route of degradation, expression of the CYP2B1-3A4CT chimera in hrd2Δ yeast led to a statistically significant 2-fold stabilization of this protein (Fig. 7), whereas the unmodified CYP2B1 protein was unaffected in this yeast strain (Fig. 3). These findings thus revealed that the incorporation of the CYP3A4 C-terminal heptapeptide was not sufficient to target the chimeric CYP2B1-3A4CT protein for 26S proteasomal degradation in a manner similar to that of the wild-type CYP3A4 protein. However, unlike the presumably coupled Ubc7p/Hrd2p dependence of the degradation of native CYP3A4 protein, no corresponding Ubc7p dependence was found for CYP2B1-3A4CT, as determined by the lack of appreciable stabilization of the chimeric protein after its expression in ubc7Δ yeast (Fig. 8). Thus, the proteasomal degradation of CYP2B1-3A4CT either involves ubiquitination by a Ub-conjugating enzyme other than Ubc7p or is independent of ubiquitination, a less likely possibility given the Hrd2p (19S cap subunit)-dependent degradation of this modified CYP2B1 protein.

The possibility that the observed proteasomal degradation of CYP2B1-3A4CT is caused by misfolding and consequent instability of the chimeric protein relative to that of the unmodified CYP2B1 was excluded by the following criteria: each P450 protein was heterologously expressed in two different yeast strains, and microsomal fractions were prepared from cells harvested at the early growth stage of culture. The spectrally detectable microsomal P450 (holoenzyme) con-
tents of CYP2B1-3A4CT and unmodified CYP2B1 (Fig. 9) were compared with their contents of immunodetectable CYP2B1 protein (Table 2). Comparable ratios of holoenzyme content/total CYP2B1-immunoreactive protein content indicated equivalent efficiencies of folding and hemoprotein assembly of both proteins in both yeast strains examined (Table 2). Furthermore, when these proteins were heterologously expressed in E. coli, a considerably higher yield of CYP2B1-3A4CT than that of the parent CYP2B1 was observed (Table 3), a finding inconsistent with a folding defect for this modified protein. Functional reconstitution of the partially purified heterologously expressed recombinant proteins using testosterone 16β-hydroxylase as a CYP2B1-selective functional probe also revealed comparable specific activities, thereby indicating no alteration in function by the CYP3A4CT modification (Table 3). When these proteins were heterologously expressed in E. coli, a considerably higher yield of CYP2B1-3A4CT than that of the parent CYP2B1 was observed (Table 3), a finding inconsistent with a folding defect for this modified protein. Functional reconstitution of the partially purified heterologously expressed recombinant proteins using testosterone 16β-hydroxylase as a CYP2B1-selective functional probe also revealed comparable specific activities, thereby indicating no alteration in function by the CYP3A4CT modification (Table 3). Because both the P450 holoenzyme spectrum and the catalytic activity are sensitive measures of the integrity of the CYP2B1 protein, it seems that the altered degradation of CYP2B1-3A4CT compared with that of the unmodified CYP2B1 is not caused by any structural change affecting the active site.

Discussion

The finding that native CYP2B1 incurs Pep4p-dependent vacuolar degradation after its heterologous expression in S. cerevisiae (Fig. 4) establishes that its proteolytic course in yeast is analogous to its normal (lysosomal) route in the rat liver. Thus, despite being an “alien” protein, CYP2B1 is degraded via the yeast vacuolar pathway rather than by the Ub-dependent 26S proteasomal system, the pathway for degradation of abnormal proteins. These findings thus further validate S. cerevisiae as a reliable model for the characterization of hepatic CYP2B1 degradation. Together with our previous findings (Murray and Correia, 2001; Murray et al., 2002), they also confirm that in yeast, as in the mammalian liver, the longer-lived P450 proteins (CYP2B1 and CYP2C11) undergo vacuolar degradation, whereas the shorter turnover proteins (CYP3A) undergo proteasomal degradation. On the other hand, mammalian liver CYP2E1, which exhibits biphasic turnover with a rapid-phase component of \( t_{1/2} \approx 7 \) h and a slow-phase component of \( t_{1/2} \approx 37 \) to 38 h, apparently is a substrate of both proteolytic systems (Ronis and Ingelman-Sundberg, 1989; Ronis et al., 1991; Tierney et al., 1992; Roberts et al., 1995; Yang and Cederbaum, 1996; Bardag-Gorce et al., 2002). The differential processing of the same P450 protein may be related to the occupancy of its active site. Therefore, CYP2E1 complexation with a substrate ligand such as ethanol or acetone stabilizes the enzyme, thereby increasing its content and converting it into a longer-lived species with a monophasic turnover with \( t_{1/2} \approx 37 \) to 38 h that is susceptible to autophagic-lysosomal sequestration. However, substrate decomplexation (e.g., by ethanol withdrawal) converts the CYP2E1 protein into a rapid turnover species that is susceptible to proteasome-inhibitor sensitive degradation (Bardag-Gorce et al., 2002). Together, these findings suggest that substrate complexation can determine the relative fraction

![Degradation of CYP2B1 and CYP2B1-3A4CT in S. cerevisiae](1465)
and interconvertability of the short- and long-lived CYP2E1 species and thus dictate the enzyme’s relative susceptibility to either of the two proteolytic pathways. Similar substrate-induced protein stabilization is also observed after metabolic intermediate complexation of dexamethasone-inducible CYP3A23 by the quasi-irreversible, mechanism-based inactivator troleandomycin (TAO).6 TAO is known to dramatically extend the half-lives of CYP3A23 apoprotein and heme moieties from 14 and 10 h to 63 and 73 h, respectively, with an attendant-marked “induction” of CYP3A23 content (Watkins et al., 1986, 1987). Whether such TAO complexation also switches the proteolytic susceptibility of CYP3A23 from predominantly proteasomal to lysosomal degradation remains to be determined. It is noteworthy, however, that in the absence of substrates, both CYP2E1 and CYP3A23 are highly prone to oxidative uncoupling and consequent oxidative damage. Thus, not surprisingly, the suppression of such oxidative uncoupling through the disruption of their prosthetic heme reduction (Zhukov and Ingelman-Sundberg, 1999; Henderson et al., 2003) or quasi-irreversible complexation of the P450 heme (Watkins et al., 1986) protects the enzyme from oxidative damage and rapid proteolysis with consequent protein stabilization. Given this possibility, it is conceivable that similar complexation of CYP2B1 with a substrate-inducer molecule and/or its relative resistance to oxidative uncoupling also account(s) for its relatively long protein half-life and its predominant targeting to lysosomal degradation in the rat liver (Masaki et al., 1987; Ronis et al., 1991). However, the above findings that 1) in the absence of any exogenously added CYP2B1 substrate, CYP2B1 in S. cerevisiae maintained in minimal medium is similarly targeted to the vacuole, the yeast lysosomal equivalent, and 2) grafting of just seven additional CYP3A4 C-terminal residues onto the CYP2B1 C terminus switches its proteolytic targeting without detectably altering the CYP2B1 protein or active site structure and/or function (Fig. 9 and Table 3) argue for the plausible existence of specific determinants that dictate the route of pro-

Fig. 6. Relative stabilization of CYP2B1-3A4CT in wt (PEP4) and pep4Δ S. cerevisiae strains. The expression plasmid used was pYES2-ADH/CYP2B1-3A4CT. For other experimental details, see Fig. 2. Values represent the mean ± S.D. of at least three individual experiments, with no statistically significant difference found between them.

Fig. 7. Relative stabilization of rat CYP2B1-3A4CT in wt and hrd2-1 mutant S. cerevisiae strains. For experimental details, see Fig. 2. Values each represent the mean ± S.D. of at least three individual experiments. *, statistically significant difference at p < 0.05 relative to the corresponding wt control.

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6 TAO-mediated CYP3A mechanism-based inactivation is “quasi-irreversible” in that the enzyme can be catalytically reactivated by ferricyanide-induced heme-iron oxidation, which dissociates the TAO-nitrene/CYP3A complex, thereby regenerating the enzyme.
teolytic degradation of the native P450 protein. The notion that CYP3A4 could contain a proteasomal determinant is consistent with the fact that in yeast, as in the liver, CYP3A4 retains its 26S proteasomal susceptibility. This is so even though the protein is almost certainly largely in its native state when so expressed, as its redox function, and hence its potential for damage inflicted by oxidative uncoupling is greatly attenuated in the absence of coexpressed P450 reductase.

The precise identity of such proteolytic determinants and/or the nature of the signals for the differential proteolytic sorting in normal P450 turnover remain to be defined. Such determinants could include degradation motifs (i.e., “degrons”) inherent to the P450 protein structure or acquired and/or unmasked as a result of protein modification associated with the P450 oxidative/catalytic function. Common post-translational modifications that predispose intracellular proteins for proteolytic removal include Ub conjugation; glutathione-protein–mixed disulfide formation; histidine, cysteine, or methionine oxidations; phosphorylation/dephosphorylation; deamidation; and glycosylation (Correia, 2003). Intrinsic structural features of target proteins required for their recognition by proteolytic systems include lysine or N-terminal residues for Ub conjugation; proline, glutamic acid, serine, and threonine/PAGE sequences for kinases/calpains and other Ca$^{2+}$-dependent proteases; “destruction boxes”; degradation motifs; “KK” motifs; C-terminal basic or acidic residues for ER degradation; and KFERQ, [G]YXXØ, [DE]XXXL[LI], and DXXLL consensus motifs for lysosomal sorting/autophagy (Rogers et al., 1986; Dice et al., 1990; Sokolik and Cohen, 1992; King et al., 1996; Johnson et al., 1998; Bonifacino and Traub, 2003).

We find it intriguing that inspection of the CYP2B1 protein sequence reveals the existence of a lysosomal sorting signal in the N-terminal region of the protein: a [DE]XXXL[LI] motif at D$_{47}$RGGGL$_{52}$, conveniently located between the predicted CYP2B1 ER-membrane anchor and cytosolic domain. No corresponding lysosomal sorting motif is present in CYP3A4, a proteasomal degraded P450 protein. Furthermore, similar sequence analysis of CYP2C11, another lysosomally degraded protein, indicated that although it contains no corresponding [DE]XXXL[LI] motif, it does carry a lysosomal sorting GYXXØ motif at G$_{79}$YEAV$_{83}$. The presence of such determinants may ensure lysosomal degradation as a “default” route for the native P450s. Whether these inherent P450 structural motifs by themselves are sufficient as lysosomal sorting determinants or whether they require additional yet-to-be-identified cellular sorting and/or trafficking machinery for their lysosomal recognition remains to be elucidated. Nevertheless, the ability of the CYP3A4 C-terminal heptapeptide to confer proteasomal susceptibility upon

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7 The dileucine [DE]XXXL[LI] lysosomal sorting signal requires an acidic residue at position −4, from the first invariant leucine of the LL/LI peptide motif, with three random residues (XXX) at intervening positions. A bona fide YXXØ lysosomal-sorting motif usually contains a glycine residue preceding the invariant tyrosine residue, two random residues (XX), followed by a bulky hydrophobic side chain (Ø) residue at +3 (Bonifacino and Traub, 2003).
TABLE 2

The ratios of microsomal CYP2B1 and CYP2B1-3A4CT holohemoprotein and total CYP2B1 immunoreactive protein expressed in S. cerevisiae

<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>P450</th>
<th>Total CYP2B1 Protein</th>
<th>P450 Content</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHY718 CYP2B1</td>
<td>27 ± 5</td>
<td>18 ± 9</td>
<td>0.76 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>RHY718 CYP2B1-3A4CT</td>
<td>30 ± 10</td>
<td>22 ± 10</td>
<td>0.70 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>RHY473 CYP2B1</td>
<td>12 ± 1</td>
<td>8 ± 2</td>
<td>0.66 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>RHY473 CYP2B1-3A4CT</td>
<td>15 ± 1</td>
<td>10 ± 4</td>
<td>0.68 ± 0.25</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 3

The relative expression yields and functional activities of CYP2B1 and CYP2B1-3A4CT in E. coli DH5x

<table>
<thead>
<tr>
<th>P450 Yield</th>
<th>Testosterone 16α-Hydroxylase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol/min</td>
<td>nmol OH T formed/nmol of P450/min</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>49.4</td>
</tr>
<tr>
<td>CYP2B1-3A4CT</td>
<td>103.2</td>
</tr>
</tbody>
</table>

In summary, the results described above indicate that just as in the rat liver, the vacuolar pathway is responsible for the degradation of CYP2B1 in S. cerevisiae, thereby validating this model for the characterization of mammalian liver P450 degradation. Furthermore, these findings also indicate that the proteolytic susceptibility of CYP2B1 can be switched from predominantly lysosomal to proteasomal degradation merely by appending seven CYP3A4 C-terminal residues, thereby suggesting that this CYP3A4 region could serve as a proteasomal degron.

Acknowledgements

We wish to gratefully thank Professor Randy Hampton (University of California, San Diego, CA) for the generous gift of the yeast strains; Professor James R. Halpert (University of Texas, Galveston, TX) for the gift of the plasmids pSW1 (encoding intact CYP2B1) and pKKCYP2B1(His)4; and Professor Eric F. Johnson (Scripps Institute, La Jolla, CA) for providing pertinent CYP3A4 structural information before its publication. We thank Dr. Ping Kang for generous assistance with the P450 purification and functional reconstitution studies.

References


In contrast, C-terminal truncation of ER proteins such as the mature cytochrome P450 holohemoprotein and total CYP2B1 immunoreactive protein expressed in S. cerevisiae.

The genotypes of the yeast strains used are described in Table 1. Yeast were grown in three separate 2L-batches and harvested at the early logarithmic growth stage (see Materials and Methods). The yeast strain RHY718 was transformed with the expression plasmid pYcDE/2B1 (CYP2B1) or pYcDE/2B1-3A4CT (CYP2B1-3A4CT), whereas the yeast strain RHY473 was transformed with plasmid pYES2 BET1 (CYP2B1) or pYES2-ADH2B1-3A4CT (CYP2B1-3A4CT) as described under Materials and Methods. Microsomes were prepared and subjected to Western immunoblotting analyses as described previously (Murray et al., 2001) with a purified liver CYP2B1 preparation used as the standard for quantification. The P450 spectral content was determined as described previously (Murray et al., 2001). The molar ratio of P450 holohemocytchrome content/total CYP2B1 immunoreactive protein content is shown.

cCYP2B1, an otherwise stable protein that is normally degraded by the lysosomes, suggests that such lysosomal determinants can be either overridden or suppressed. However, the documentation of Ub-dependent proteasomal degradation of the cumene hydroperoxide-inactivated CYP2B1 in vitro (Korsmeyer et al., 1999), reveals that the enzyme also harbors ubiquitination/proteasomal determinants that can be unmasked by its structural and functional inactivation.

The precise significance of the CYP3A4 C terminus (D497GTVSGA503) as a potential proteasomal targeting determinant is presently unclear. The recently reported crystal structures of CYP3A4 reveal that this region is unstructured and entirely dispensable for correct folding6 (Williams et al., 2004; Yano et al., 2004). Thus, it is conceivable that this unstructured CYP3A4 region serves as a recognition signal for degradation by engaging either a 19S cap subunit before protein unfolding and/or insertion into the proteasomal catalytic barrel or a yet-to-be-identified Ub ligase that ubiquitinates the protein before its delivery to the 26S proteasome (Pickart and Cohen, 2004). It is intriguing in this context that the proteasomal targeting and subsequent proteolytic processing of ornithine decarboxylase, a 461-residue long protein, is also dependent on a flexible, unstructured 37-residue C-terminal region as a recognition signal (Zhang et al., 2003, 2004). CYP34A and the extreme C-terminal pentapeptide (A145RINV361) in this ornithine decarboxylase region are apparently indispensable for its insertion into the proteasomal catalytic chamber before its degradation. Furthermore, this 37 amino acid C-terminal tail can function as a proteasomal targeting signal when appended to other proteins (Zhang et al., 2003). The possibility of the CYP3A4 C-terminal heptapeptide similarly functioning as a degron is particularly compelling, not only because it conferred proteasomal susceptibility on CYP2B1, but also because, as discussed above, it may enable CYP3A4 despite its functional sluggishness in yeast to retain its preferential degradation by the proteasome. Studies are currently underway to determine 1) which, if any, of the CYP3A4 heptapeptide (D497GTVSGA503) residues are essential for a functional proteasomal degron; 2) whether this appendage could similarly switch the targeting of another bona fide lysosomal substrate; 3) whether its excision from the CYP3A4 C terminus would stabilize the enzyme by rendering it nonsusceptible to proteasomal degradation; and 4) whether it could be replaced by any other similarly unstructured peptide.

Address correspondence to: Dr. M. A. Correia, Department of Cellular and Molecular Pharmacology, Box 0450, University of California, San Francisco, CA 94143-0450. E-mail: mariac@itsa.ucsf.edu

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