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# Native CYP2C11: Heterologous Expression in *Saccharomyces cerevisiae* Reveals a Role for Vacuolar Proteases Rather Than the Proteasome System in the Degradation of This Endoplasmic Reticulum Protein

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## ABSTRACT

Cytochromes P450 (P450s) are hemoprotein enzymes committed to the metabolism of chemically diverse endo- and xenobiotics. They are anchored to the endoplasmic reticulum (ER) membrane with the bulk of their catalytic domain exposed to the cytosol, and thus they constitute excellent examples of integral monotopic ER proteins. Physiologically they are known to turn over asynchronously, but the determinants that trigger their proteolytic disposal and the pathways for such cellular disposal are not well defined. We recently showed that CYP3A4, the dominant human liver drug-metabolizing enzyme, and its rat liver orthologs undergo ubiquitin-dependent 26S proteasomal degradation not only after suicide inactivation, but also when CYP3A4 is expressed in *Saccharomyces cerevisiae*, presumably in its "native" form. The latter findings, obtained by the use of strains either with compromised proteasomal degradation of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) or deficient in ubiquitin-conjugating enzymes (Ubc; UBC), revealed that this native monotopic P450 enzyme, in common with the polytopic HMGR, required the function of certain *HRD* (HMGR degradation) and *UBC* genes. In this study, we examined the degradation of CYP2C11, a male rat liver–specific P450, by heterologous expression in *S. cerevisiae* under comparable conditions. We report that unlike CYP3A4 and HMGR, the degradation of CYP2C11 in *S. cerevisiae* is independent of either *HRD* or *UBC* gene function, but it is largely dependent on vacuolar (lysosomal) proteolysis. These findings with two monotopic ER hemoproteins, CYP2C11 and CYP3A4, and the polytopic ER protein HMGR attest to the remarkable mechanistic diversity of cellular proteolytic disposal of ER proteins.

Endoplasmic reticulum (ER) proteins are reportedly subject to a quality control system that marks unassembled and/or misfolded residents for degradation by the cytosolic ubiquitin (Ub)-dependent 26S proteasome system (Le et al., 1992; Adeli, 1994; Ward et al., 1995; Qu et al., 1996; Werner et al., 1996; Wiertz et al., 1996; Hill and Cooper, 2000). This also is the mechanism by which the cellular levels of some tightly regulated ER proteins such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), the rate-limiting enzyme in sterol biosynthesis, are modulated (Hampton and Rine, 1994; Hampton et al., 1996; McGee et al., 1996; Wilhovsky et al., 2000). The degradation of HMGR Hmg2p has been well characterized in *Saccharomyces cerevisiae,* and three genes—*HRD1*, *HRD2,* and *HRD3*, so termed for HMGR degradation (Hampton and Rine, 1994; Hampton et al., 1996)—have been identified as essential. Hrd2p is a subunit of the 19S cap of the 26S proteasome (Hampton et al., 1996). Hrd1p, also known as Der3p, is an integral ER protein that is absolutely required for the degradation of certain luminal proteins, such as carboxypeptidase Y (CPY), as well as the mutated ER-translocon protein Sec61–2p (Sommer and Wolf, 1997; Bordallo et al., 1998; Plemper et al., 1999) and has been characterized as an ER-associated Ub-ligase (E3). There is believed to be an intimate association between Hrd1p and the other HRD gene product, Hrd3p. The Hrd1p-Hrd3p complex–mediated HMGR ubiquitination is also dependent on an ER-associated soluble Ub-conjugating enzyme Ubc7p, but not Ubc6p, an integral ER protein (Plemper and Wolf, 1999; Wilhovsky et al., 2000). Thus, Ubc7p and the Hrd1p-Hrd3p

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**ABBREVIATIONS:** CPY, carboxypeptidase Y; P450, cytochrome P450; DDEP, 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine; ER, endoplasmic reticulum; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HRD, 3-hydroxy-3-methylglutaryl-CoA reductase degradation; OR, NADPH-cytochrome P450 oxidoreductase; Ub, ubiquitin; Ubc, Ub-conjugating enzyme; ura, uracil; wt, wild-type; TBS, Tris-buffered saline.

complex are collectively responsible for the ubiquitination and subsequent delivery of the polytopic HMGR to the 26S proteasome (Hampton et al., 1996; Sommer and Wolf, 1997; Bays et al., 2000; Gardner et al., 2000). Ubc7p also has been shown to be involved in the degradation of several other proteins such as CPY*, Sec61–2p, *Deg1* degron-equipped mat$\alpha$2 transcriptional repressor, and *Deg1*-Hmg1p and *Deg1*-Hmg2p fusion proteins (Chen et al., 1993; Hampton and Bhakta, 1997; Plemper and Wolf, 1999; Wilhovsky et al., 2000), in many of these instances with the functional assistance of an integral ER protein Cue1p (Biederer et al., 1997).

Although the targeting of aberrant proteins for degradation by the Ub-dependent 26S proteasomal system is understandable, it is less clear whether resident proteins are also physiologically degraded through this pathway. Typical examples of such residents are the monotopic N-terminally anchored hemoprotein enzymes of the mammalian liver cytochrome P450 (P450) family (De Lemos-Chiarandini et al., 1987; Monier et al., 1988; Kemper and Szczesna-Skorupa, 1989; Sato et al., 1990; Black et al., 1994), which are engaged in the oxidation, reduction, and/or dehydrogenation of a host of structurally and chemically diverse endo- and xenobiotics (Ortiz de Montellano, 1995). Although most of these catalytic cycles are productive, in the presence of certain substrates that generate reactive intermediates, the enzymes can incur mechanism-based suicide inactivation (Ortiz de Montellano and Correia, 1995). We have recently shown that one form of such inactivation can modify the protein and mark it for rapid proteolytic disposal via the Ub-dependent 26S proteasomal system (Correia et al., 1992; Korsmeyer et al., 1999; Wang et al., 1999). Because such suicide inactivation of P450s markedly disrupts their normal structure, it is not surprising that such an insult would qualify them as "aberrant" and substrates for proteasomal degradation. However, we have recently shown that a "native"[2] P450, CYP3A4, the major human liver drug-metabolizing isoform, when expressed in *S. cerevisiae*[3] also uses the Ub-dependent 26S proteasomal degradation pathway and is dependent on Ubc7p and Hrd2p, and to a lesser extent Hrd3p (Murray and Correia, 2001). To determine whether this degradation was a unique feature of CYP3A4, or whether it reflected the fact that albeit "native and unmodified," this enzyme was nevertheless an "abnormal" protein to yeast, we examined another monotopic ER-bound P450, male rat liver–specific CYP2C11 in these yeast strains. Our findings reveal that, unlike CYP3A4, CYP2C11 is not a substrate of the Ubc7-dependent 26S proteasomal degradation in yeast, but it is probably a substrate of vacuolar proteases, the yeast equivalent of lysosomal degradation. These findings with two structurally related members of the P450 hemoprotein family of comparable normal protein half-lives further attest to the mechanistic diversity and complexity of ER protein degradation.

[2] By use of the term "native," we merely mean that the P450 protein structure has not been intentionally modified by prosthetic heme fragments or other chemically reactive species.

[3] As discussed in detail previously (Correia, 1991), degradation studies of long-lived hepatic P450s such as CYP2C11 cannot be conducted either in freshly isolated hepatocytes because of the limited viability of the latter (≈5–6 h) or in cultured hepatocytes because of P450 instability (i.e., accelerated loss of heme). This led to our use of *S. cerevisiae* as a model. The validity of this model for examination of mammalian protein degradation has been established in the literature (Murray and Correia, 2001) and was further substantiated by the recent documentation of murine homologs of yeast Ubc6p and Ubc7p (Tiwari and Weissman, 2001).

## Experimental Procedures

**Materials.** General reagents were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma (St. Louis, MO). Yeast lytic enzyme from *Arthrobacter luteus* was obtained from ICN Pharmaceuticals (Costa Mesa, CA). Electrophoresis and transblotting reagents were obtained from Bio-Rad (Hercules, CA). Microbiological reagents were obtained from Difco (Detroit, MI). Bicinchoninic acid protein assay reagent and SuperSignal chemiluminescent substrate for horseradish peroxidase were from Pierce Chemical (Rockford, IL). Polyclonal rabbit IgGs to purified recombinant CYP2C11 were raised commercially (Research Genetics, Huntsville, AL) and purified by protein A chromatography. Mouse monoclonal anti-myc antibody 9E10 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-Sec61p and anti-Sec63p IgGs were gifts from Professor Peter Walter (University of California, San Francisco, San Francisco, CA) and Professor Randy Schekman (University of California, Berkeley, Berkeley, CA).

**Yeast Strains.** As previously in our studies of CYP3A4 degradation (Murray and Correia, 2001), the *S. cerevisiae* strains used comprise isogenic sets, one known to be deficient in HMGR degradation (*hrd*; Hampton and Rine, 1994; Hampton et al., 1996; Wilhovsky et al., 2000), another deficient in Ub conjugation (*ubc*; Hampton and Bhakta, 1997; Wilhovsky et al., 2000), and another isogenic pair, one member of which possesses a complete disruption of the *PEP4* reading frame *(pep4Δ)* [and has been proven deficient in CPY degradation by vacuolar proteases (Hampton and Rine, 1994)], were kindly donated by Professor Randolph Hampton (University of California, San Diego, San Diego, CA). The only difference in this study is that the *hrd1*-deficient yeast strain (RHY609) is a tryptophan rather than a leucine auxotroph. The *pep4*Δ phenotype was confirmed by the lack of functional CPY determined by the *N*-acetyl-DL-phenylalanine $\beta$-naphthyl ester esterase and *N*-benzoyl-L-tyrosine *p*-nitroanilide amidase activities as described previously (Murray and Correia, 2001). The strains used are listed in Table 1.

**Plasmids.** Plasmid pD2M1 [a generous gift from Drs. M. Sakaguchi and T. Omura (Kyushu University, Fukuoka, Japan) (Hayashi et al., 1988)] is a TRP-marked 2-$\mu$ plasmid with the rat CYP2C11 cDNA under the control of the yeast ADH1 promoter. Plasmid pYcDE-2/luc used as the vector control was constructed by replacing the CYP2C11 cDNA with that for modified firefly luciferase from pSP-luc+ (Promega, Madison, WI). The URA-marked vector, pYES2, was obtained from Invitrogen (Carlsbad, CA), and the CYP2C11 cDNA from pD2M1 was introduced as an *Eco*RI fragment. Plasmid pHHCSA65 containing a 1.5-kilobase cDNA fragment of the human 18S rRNA was obtained from American Type Culture Collection (Manassas, VA).

**Yeast Cell Transformation.** Cell transformation was achieved as described previously (Murray and Correia, 2001). The presence of the kanMX gene was tested by culture at 30°C in YePD (2% Bactopeptone, 1% yeast extract, 2% glucose) containing 0.2 g of active G418 per liter. Transformed yeast cells were otherwise grown at 30°C in SD or SG medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose or galactose) with appropriate supplements. Cells were harvested during logarithmic growth phase at an optical density of 1.0, or after "stationary chase," generally, 10 to 12 h after reaching an optical density of 0.5 at 600 nm (Hampton et al., 1996), except that in the studies with *pep4*Δ yeast, cells were harvested up to 18 h after reaching this point.

**Microsomal Subfraction Preparation.** Microsomes were prepared by differential centrifugation from sonicates of yeast spheroplasts and stored at −80°C in 0.25 M potassium phosphate buffer, pH 7.25, containing 30% (v/v) glycerol, exactly as described previously (Murray and Correia, 2001). Spectrally detectable P450 content was monitored as described previously (Hayashi et al., 1988). Protein concentrations of these fractions were determined by use of the bicinchoninic acid method after precipitation with 5% sulfuric

acid in methanol, followed by acetone and ethanol washes and solubilization in 1 M NaOH.

**CYP2C11 Western Immunoblotting Analysis.** Aliquots of microsomal protein (1 $\mu$g) and purified CYP2C11 were subjected to denaturing electrophoresis under reducing conditions in 9% polyacrylamide minigels followed by electroblotting onto nitrocellulose at 100 V for 1 h. After blocking with 3% gelatin in Tris-buffered saline (TBS), pH 7.5, for at least 1 h, membranes were exposed to primary antibody (rabbit anti-CYP2C11 IgGs) diluted in TBS containing 0.05% Tween 20 and 1% gelatin for at least 2 h. Membranes were then washed and treated with peroxidase-labeled second antibody (goat anti-rabbit IgGs) and then washed, and the signal was visualized by immersion of the filter in chemiluminescent substrate for 5 min followed by exposure to Kodak BIOMAX MR film (Eastman Kodak, Rochester, NY). The signals were quantified by a scanning densitometer and UN-SCAN-IT software (Silk Scientific, Orem, UT) running on a Macintosh G3 personal computer. The corresponding expression of Sec61p and Sec63p was monitored immunochemically exactly as described previously (Murray and Correia, 2001), as were the slot-blotting analyses of myc-tagged Hmg2p.

**RNA Analyses.** Total RNA was extracted during the logarithmic growth phase and subjected to slot-blotting analyses, as described previously (Murray and Correia, 2001). CYP2C11 cDNA probe was labeled with [$\alpha$-$^{32}$P]dCTP using the random primer method, purified by chromatography on Sephadex G-50, and then denatured by heating at 95°C for 5 min before hybridization. The blots were washed with 0.1× SSC/0.1% SDS at 50°C for 1 h and then exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). The signal was normalized after stripping the P450 probe from the membrane by subsequent hybridization with the 18S rRNA probe. The signals were quantified with a Storm Imager (Molecular Dynamics) using ImageQuant 1.2 software. As shown previously (Murray and Correia, 2001), pretreatment of samples with RNase-free DNase I had no effect on signal intensity, whereas pretreatment with RNase completely prevented any signal from being detected.

**Statistical Analyses.** Statistical analyses were conducted by use of the two-tailed Student's $t$ test, and a probability of $p < 0.05$ was considered statistically significant.

## Results

**CYP2C11 Expression in Wild-Type (wt) and *hrd*-Deficient Yeast.** As with CYP3A4 (Murray and Correia, 2001), the immunochemically detectable microsomal CYP2C11 content in wt and *hrd1, hrd2-*, and *hrd3*-deficient yeast harvested at early stages of culture (i.e., during logarithmic growth phase, OD ≈ 1.0), revealed comparable expression of this enzyme in all of the four yeast strains, indicating equivalent transcriptional/translational efficiency of the CYP2C11 cDNA (Fig. 1). Corresponding RNA analyses yielded comparable CYP2C11-specific mRNA content in all yeast strains at this stage of culture, thereby corroborating the evidence for equivalent transcriptional efficiency from the plasmid (data not shown). However, contrary to the findings observed with the CYP3A4 protein (Murray and Correia, 2001), no significant differences in CYP2C11 content were observed between the wt and *hrd1-*, *hrd2-*, and *hrd3*-deficient yeast during the stationary growth phase of the culture, thereby revealing that CYP2C11 degradation was presumably independent of all three *HRD* genes (Fig. 1). That CYP2C11 was indeed degraded in these cells at that time was documented by the appreciable decrease [average losses of ≈54 ± 18% ($n$ = 6; $p < 0.05$)] in the immunochemically detectable CYP2C11 protein levels from its corresponding levels at the earlier stages of culture (Fig. 1). The yeast strains transformed with the control vector yielded no immunochemically detectable CYP2C11 (Fig. 1), nor was there any RNA hybridizing with the CYP2C11 probe (data not shown), thereby confirming the specificity of both the cDNA probe and the antibodies used for CYP2C11 immunodetection. Because of these results, and because the yeast strains were not all identical with those used previously by us (Murray and Correia, 2001; Table 1), we reexamined their Hmg2p content in parallel by use of immunoblotting analyses of the stably expressed myc-tagged Hmg2p (data shown in the composite Fig. 2). These findings revealed that during the stationary phase of yeast growth, Hmg2p was greatly and significantly stabilized in all three *hrd1-*, *hrd2-*, and *hrd3*-deficient yeast strains. This was in marked contrast to its substantial loss in the wt strain, thereby essentially confirming previous observations (Hampton et al., 1996; Wilhovsky et al., 2000) and ours in CYP3A4-transformed yeast (Fig. 2; Murray and Correia, 2001) and validating the phenotypes of these yeast strains under these particular growth conditions.

**CYP2C11 Degradation in wt, *ubc6-*, *ubc7-*, and *ubc6/7*–Deficient *S. cerevisiae*.** Most proteins targeted to the 26S proteasomal degradation are usually polyubiquitinated. However, not all polyubiquitinated proteins are targeted for such degradation, because some are actually degraded by the lysosomal pathway (Hershko and Ciechanover, 1998). We therefore sought to determine whether, in common with Hmg2p and CYP3A4, the degradation of native CYP2C11 was dependent on ubiquitination by either of the two ER-associated Ubcs, Ubc6p and Ubc7p. To explore this particular possibility, we transformed wt yeast and strains deficient in Ubc6, Ubc7, or both Ubc6 and Ubc7 (Ubc6/7 double mutant) with the CYP2C11 expression vector pD2M1 or with the control vector (Fig. 3). Once again, at the early stages of

TABLE 1

Yeast strains used in our studies

Strains RHY718, -609, -925, and -749 are an isogenic set and correspond to *wt* and *hrd1-*, *hrd2-*, and *hrd3*-deficient *S. cerevisiae* mutants. RHY1166, -1596, -1603 and 1–604 are also an isogenic set and correspond to *wt* and *ubc6-*, *ubc7-*, and *ubc6/7*-deficient *S. cerevisiae* mutants. Strains RHY473 and -106-4 correspond to *PEP4* and *pep4*Δ. The method of their construction has been described previously (Hampton and Rine, 1994; Hampton et al., 1996; Wilhovsky et al., 2000).

| Strain | Genotype |
|---|---|
| RHY718 | *MATα ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2Δ trp1Δ ura3–52 + pRH244 (URA3,6mycHMG2)* |
| RHY609 | *MATα ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 LEU trp1::hisG ura3–52::6MycHMG2 hrd1::URA3* |
| RHY925 | *MATα ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2Δ trp1Δ URA3::6MycHMG2 hrd2–1* |
| RHY749 | *MATα ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2Δ trp1Δ ura3–52::6MycHMG2 hrd3::URA3* |
| RHY1166 | *MATα ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2Δ trp1Δ ura3–52::MycHMG2* |
| RHY1596 | *MATα ade2–101 met2 his3Δ200 hmg2::HIS3 lys2–801 hmg1::LYS2 leu2Δ trp1Δ ura3–52::MycHMG2 ubc6::KanMX* |
| RHY1603 | *MATα ade2–101 met2 his3Δ200 hmg2Δ-4 lys2–801 hmg1::LYS2 leu2Δ trp1Δ ura3–52::MycHMG2 ubc7::HIS3* |
| RHY1604 | *MATα ade2–101 met2 his3Δ200 hmg2Δ-4 lys2–801 hmg1::LYS2 leu2Δ trp1Δ ura3–52::MycHMG2 ubc6::KanMX ubc7::HIS3* |
| RHY473 | *ade2–101 met2 his3Δ200 lys2–801 ura3–52* |
| RHY106-4 | *ade2–101 met2 his3Δ200 lys2–801 ura3–52 pep4Δ* |

logarithmic cell growth, CYP2C11 was equivalently expressed in all four strains, thereby revealing comparable transcriptional and translational efficiencies in all four strains, as confirmed independently by the corresponding CYP2C11 mRNA analyses conducted at that time (data not shown). However, at later stages of yeast culture (Fig. 3), in contrast to our findings with CYP3A4 (Fig. 4; Murray and Correia, 2001) and those with Hmg2p (Hampton et al., 1996; Wilhovsky et al., 2000), no differences in the relative stability of CYP2C11 were observed between the four yeast strains, thereby excluding a requirement for ER-associated Ubc6p and -7p in the degradation of this native ER protein. Parallel immunoblotting analyses of Hmg2p in these yeast strains confirmed the findings of Hampton and others (Hampton and Bhakta, 1997; Wilhovsky et al., 2000) that Ubc7p was critically important in the 26S proteasomal degradation of this polytopic ER protein (Fig. 4), thereby validating the phenotypes of the yeast strains.

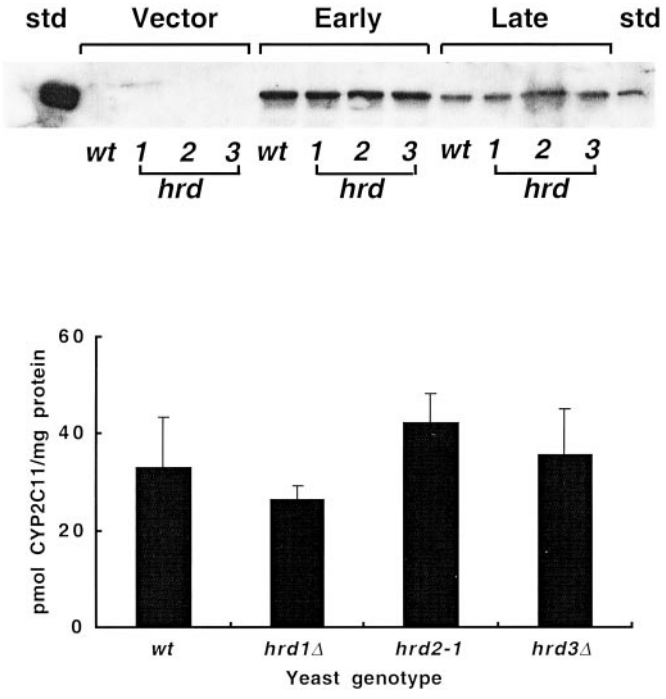



**Fig. 1.** Relative stabilization of CYP2C11 at early and late culture stages in wt and *hrd*-deficient *S. cerevisiae* strains. Yeast strains were transformed with CYP2C11 vector (pD2M1) or the empty vector (pYcDE-2-Luc). Culture aliquots were taken at early or late stages (described under *Experimental Procedures*), and microsomal CYP2C11 content was determined by Western immunoblotting (described under *Experimental Procedures*). A prototypic experiment with microsomes pooled from at the least three different individual yeast cultures is depicted (top). The corresponding data (mean ± S.D.) obtained from densitometric quantitation of individual immunoblots from at the least three separately transformed yeast cultures at late stages are shown (bottom).

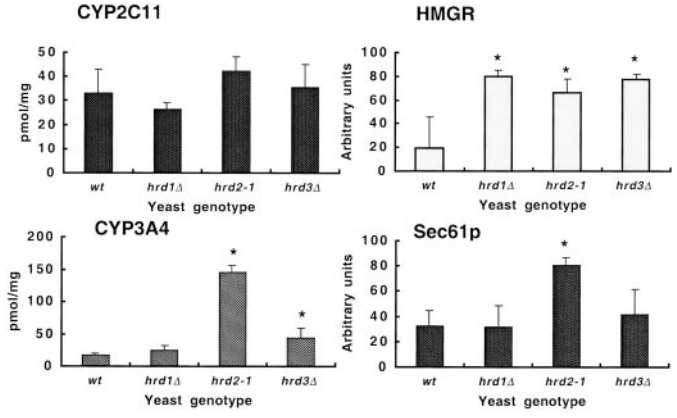



**Fig. 2.** Relative stabilization of CYP2C11 and three other ER proteins at late culture stages in wt and *hrd*-deficient *S. cerevisiae* strains. Right, relative Hmg2p and Sec61p contents were monitored in parallel with CYP2C11 in microsomes obtained from late stages of culture of pD2M1 transformed wt and *hrd*-deficient yeast strains depicted in Fig. 1. Left, microsomal CYP2C11 (Fig. 1) and CYP3A4 content (Murray and Correia, 2001) in wt and *hrd*-deficient yeast strains are depicted for direct comparison. Values represent mean ± S.D. of at the least three separate experiments. The bars marked with an asterisk indicate statistically significant differences at $p < 0.05$ relative to the corresponding wt controls.

**Diversity of ER-Protein Degradation.** Such mechanistic differences and diversity in ER-protein degradation can be further appreciated by parallel immunoblotting analyses of endogenously expressed Sec61p (shown) and Sec63p (not shown), two postulated components of the ER-translocon (Sommer and Wolf, 1997; Bordallo et al., 1998; Plemper et al., 1999; Plemper and Wolf, 1999), in the wt, *hrd*-deficient, and *ubc*-deficient yeast strains used above (Figs. 2 and 4). The findings obtained previously with CYP3A4 (Murray and Correia, 2001) and above with CYP2C11 and Hmg2p in all these strains are included for direct comparison and corresponding appreciation of this diversity (Figs. 2 and 4). Sec61p showed an approximately 2-fold stabilization in *hrd2*-deficient yeast strains and a marginal stabilization in *ubc6/7*-deficient

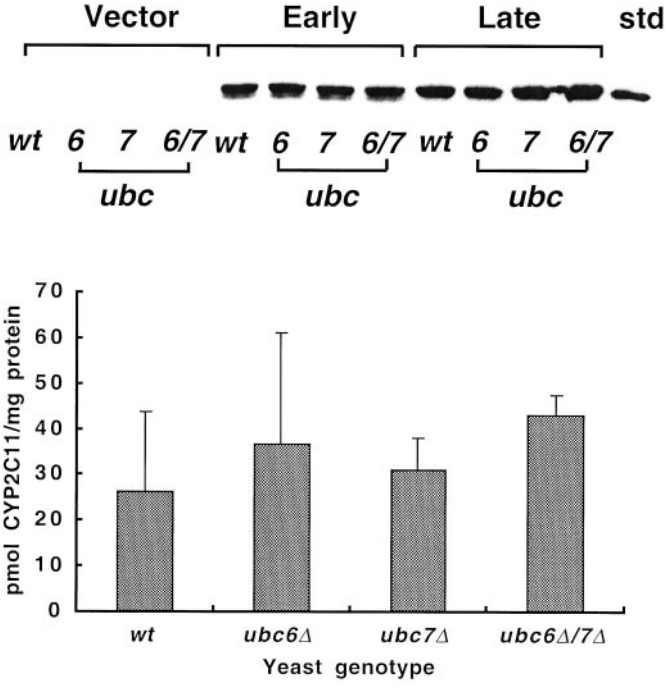



**Fig. 3.** Relative CYP2C11 stabilization at early and late culture stages in wt and *ubc6-*, *ubc7-*, and *ubc6/7*-deficient *S. cerevisiae* strains. Yeast strains were transformed with CYP2C11 vector (pD2M1) or the empty vector (pYcDE2-luc). Culture aliquots were taken at early or late stages (described under *Experimental Procedures*), and microsomal CYP2C11 content were determined by Western immunoblotting (described under *Experimental Procedures*). A prototypic experiment with microsomes pooled from at the least three different individual yeast cultures is depicted (top). The corresponding data (mean ± S.D.) obtained from densitometric quantitation of individual immunoblots from at the least three separately transformed yeast cultures at late stages are shown (bottom).

strains. However, Sec63p also showed a 2-fold stabilization in *hrd2*-deficient yeast strains, and as in the CYP3A4 studies (Murray and Correia, 2001), only a marginal stabilization in *ubc7*- and *ubc6*/*7*-deficient yeast was observed (data not shown).

**CYP2C11 Degradation in a *pep4*Δ Yeast Strain.** Certain P450s (the phenobarbital-inducible CYP2B1 and the acetone/ethanol-inducible CYP2E1) along with their catalytic cohort, NADPH-cytochrome P450 oxidoreductase (OR), another integral ER protein, reportedly undergo lysosomal degradation in rat liver cells (Masaki et al., 1987; Ronis et al., 1991). Thus, it was conceivable that CYP2C11 could follow a similar route. To examine this possibility, we transformed a yeast strain with a complete disruption of the *PEP4* reading frame *(pep4Δ)* with a plasmid expressing CYP2C11 (pYES2/CYP2C11). The *pep4*Δ phenotype of this yeast strain has been confirmed by its documented functional deficiency in the posttranslational processing of the vacuolar enzyme CPY to its mature vacuolar form (Hampton and Rine, 1994), and reconfirmed by us as described under *Experimental Procedures*. The yeast strain with intact *PEP4*-dependent vacuolar function was used as the corresponding control (wt). As in previous studies, both the *PEP4*-dependent and *pep4*Δ strains were also transformed with the control vector (pYES2), and the results confirmed the specificity of the mRNA probe and CYP2C11 immunodetection. Transformation of the wt and *pep4*Δ strains with the CYP2C11 expression vector revealed comparable levels of CYP2C11 at the early stages of culture (not shown), with an approximate 2-fold stabilization of CYP2C11 in the *pep4*Δ strain observed at the later stages relative to the corresponding levels in wt yeast equipped with the fully functional vacuolar proteases (Fig. 5, top). Essentially, similar findings were obtained when a different plasmid bearing CYP2C11 under the control of the yeast ADH1 promoter was used for transformation of these yeast strains, thereby revealing that CYP2C11 behavior was independent of the means of expression and was relatively stabilized in yeast with compromised vacuolar function (results not shown). This disparity in CYP2C11 protein expression between the two strains could not be explained by differences in levels of transcription because the *pep4*Δ strain actually exhibited somewhat less CYP2C11 mRNA than the wt counterpart (Fig. 5, bottom).

To establish whether other ER membrane-bound proteins were similarly affected by the functional deficiency of *PEP4*-dependent vacuolar protease, we determined the microsomal levels of Sec61p and Sec63p in these CYP2C11-transformed wild-type and *pep4*Δ yeast strains. Surprisingly, a dramatic Sec61p stabilization (Fig. 6, top) and a less pronounced, albeit statistically significant, Sec63p stabilization (Fig. 6, bottom) were observed in the *pep4*Δ strain relative to the corresponding wild-type levels, thereby revealing that the normal turnover of these components of the ER translocon was apparently dependent on both the 26S proteasome (Hrd-2p; Fig. 2) and *PEP4*-dependent vacuolar proteases (Fig. 6).

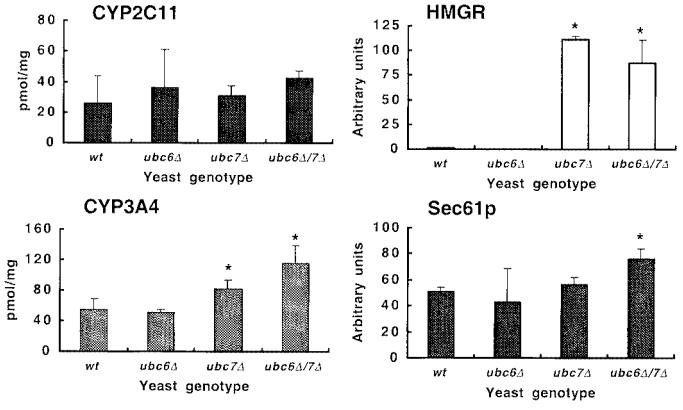



**Fig. 4.** Relative stabilization of CYP2C11 and three other ER proteins at late culture stages in wt and *ubc6*-, *ubc7*-, and *ubc6*/*7*-deficient *S. cerevisiae* strains. Right, relative Hmg2p and Sec61p content was monitored in parallel in microsomes obtained from late stages of culture of pD2M1-transformed wt and *ubc6*-, *ubc7*-, and *ubc6*/*7*-deficient yeast, depicted in Fig. 3. Left, relative microsomal CYP2C11 (Fig. 3) and CYP3A4 content (Murray and Correia, 2001) in wt and *ubc6*-, *ubc7*-, and *ubc6*/*7*-deficient yeast strains are depicted for direct comparison. Values represent mean ± S.D. of at the least three separate experiments. The bars marked by an asterisk indicate statistically significant differences at $p < 0.05$ relative to the corresponding wt controls.

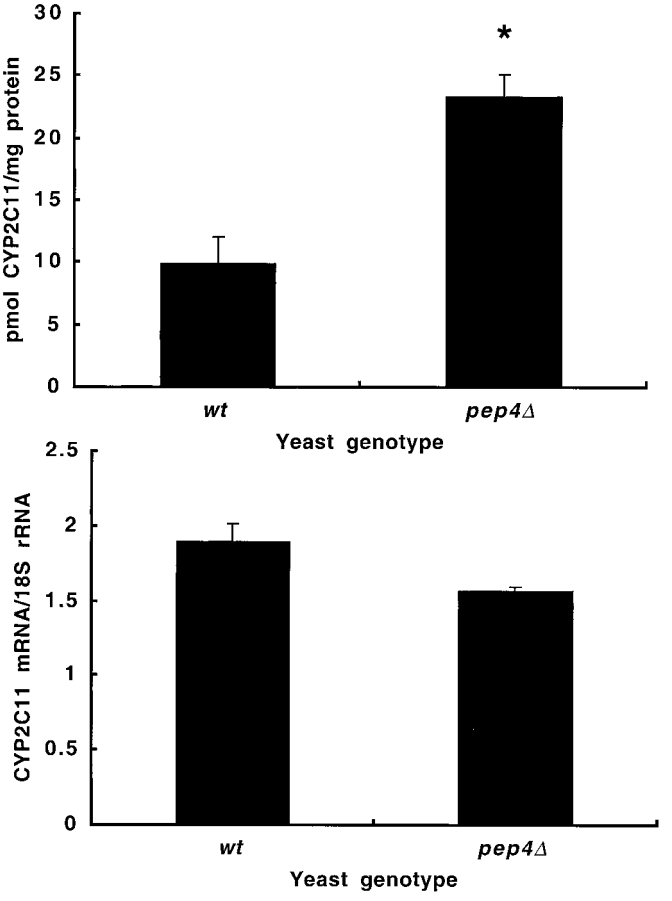



**Fig. 5.** Relative CYP2C11 stabilization at early and late stages of *pep4*Δ *S. cerevisiae* culture. Top, CYP2C11 content was monitored in microsomes obtained from pYES2/CYP2C11–transformed wt and *pep4*Δ yeast strains at approximately 18-h stages of culture. Yeast transformed with vector (pYES2) control alone were harvested in parallel at those times. Note: comparable CYP2C11 stabilization was observed between 12 and 18 h of chase period. Bottom, corresponding CYP2C11 mRNA analyses conducted with total RNA isolated at approximately 12 h (early stages) are shown. Values represent mean ± S.D. of at the least three separately cultured yeast experiments. The bar marked by an asterisk indicates statistically significant differences at $p < 0.05$ relative to the corresponding wt control.

## Discussion

The above findings in *S. cerevisiae* clearly indicate that the degradation of expressed "native" male rat liver–specific microsomal CYP2C11, unlike that of the "native" human liver CYP3A4 (Murray and Correia, 2001), yeast Hmg2p (Hampton and Rine, 1994; Hampton et al., 1996; Wilhovsky et al., 2000), and Sec61p (Bordallo et al., 1998; Plemper et al., 1999; Plemper and Wolf, 1999), is independent of the function of the *HRD* and *UBC* genes studied. The possibility remains of course that CYP2C11 is ubiquitinated either by Ubc6p and/or Ubc7p, but that such ubiquitination is not essential for its degradation, or by Ubcs other than the ER-associated Ubc6p and Ubc7p, as shown recently with Vph1p (Hill, and Cooper, 2000). One such Ubc could be Ubc1p, identified recently as an E2 enzyme participating in conjunction with the Hrd1p-RING-H2–dependent Ub-ligase in ER-protein degradation (Bays et al., 2000). Nonetheless, marked differences apparently exist in the relative importance of the 26S proteasome in the degradation of two structurally related integral ER proteins (CYP3A4 and CYP2C11). These differences, together with the findings of Hmg2p, underscore the mechanistic diversity in the ER protein degradation and the underlying differential reliance of each protein on the *HRD* gene function.

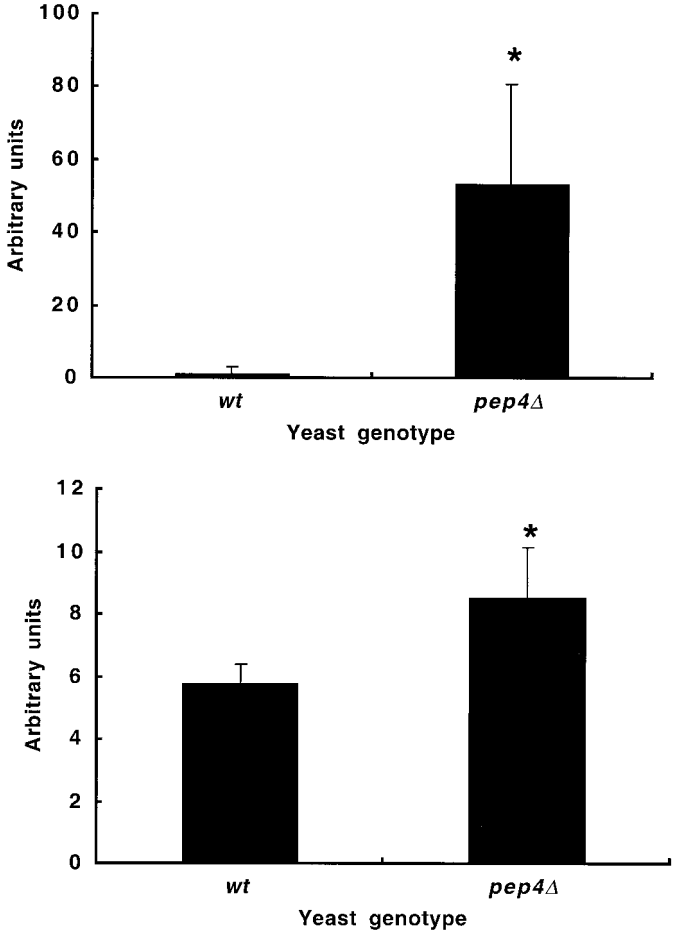



**Fig. 6.** Relative microsomal content of Sec61p and Sec63p at late stages of pYES2/CYP2C11–transformed pep4Δ *S. cerevisiae* culture. Top, relative Sec61p content was monitored immunochemically (as described under *Experimental Procedures*) in microsomes obtained from pYES2/CYP2C11-transformed wt and *pep4*Δ yeast strains at "late" (≈18 h) stages of culture (Fig. 6). Bottom, relative Sec63p content was monitored immunochemically (as described under *Experimental Procedures*) in microsomes obtained from pYES2/CYP2C11–transformed wt and *pep4*Δ yeast strains at "late" (≈18 h) stages of culture (Fig. 6). Values represent the mean of at the least three separately cultured yeast experiments. The bars marked by an asterisk indicate statistically significant differences at $p < 0.05$ relative to the corresponding wt controls.

Furthermore, our findings also reveal that CYP2C11 degradation in *S. cerevisiae* requires intact *PEP4*-dependent vacuolar function. Thus "native, unmodified" CYP2C11 uses the lysosomal route for its degradation rather than the Ub-dependent 26S proteasome pathway used by its structurally related monotopic ER-cohort CYP3A4 or the polytopic ER-protein HMGR. In this respect, it is similar to some other integral ER proteins: its structurally related hemoproteins CYP2B1 and CYP2E1, and the flavoprotein OR (Masaki et al., 1987; Ronis et al., 1991). Indeed, electron microscopic analyses with immunodetection of liver cells from rats treated in vivo with the serine protease inhibitor leupeptin, reveal "lysosomal constipation" and consequent accumulation of CYP2B1 and OR (Masaki et al., 1987). Both CYP2B1 and OR proteins share a relatively long half-life [$t_{1/2}$ = 20–37 h and 29–35 h, respectively (Shiraki and Guengerich, 1984; Watkins et al., 1987; Correia, 1991)] and thus qualify as long-lived cellular proteins, which are usually considered to be the normal substrates of lysosomal degradation. Native, unmodified CYP2E1, on the other hand, apparently undergoes a biphasic turnover (Song et al., 1989). A fraction has a much shorter apparent half-life ($t_{1/2}$ = 7 h; Song et al., 1989), consistent with its degradation by the proteasomal pathway in intact cells, although whether this specifically involves the 20S or 26S proteasome is debatable (Roberts et al., 1995; Roberts, 1997; Yang and Cederbaum, 1997). Furthermore, the purified protein can also be ubiquitinated in vitro, another indication of its plausibility as a 26S proteasomal substrate (Banerjee et al., 2000). The CYP2E1 fraction with the longer half-life ($t_{1/2}$ = 37 h) is probably the CYP2E1 pool that is committed to lysosomal degradation (Ronis et al., 1991). The half-life of CYP2C11 protein of approximately 20 ± 3 h in intact rats is not that different from that of CYP3A (10–20 h; Shiraki and Guengerich, 1984; Watkins et al., 1987; Correia, 1991) and qualifies it as a protein of an intermediate life span. Yet the nature of the structural and/or molecular determinants that commit it to proteolytic degradation by the lysosomal route rather than the 26S proteasomal route is unclear. In this context it is noteworthy that CYP2C11 shares 51.4 and 55.6% sequence identities with CYP2B1 and CYP2E1, respectively, but only approximately 24% with CYP3A23, the major rat liver CYP3A protein.

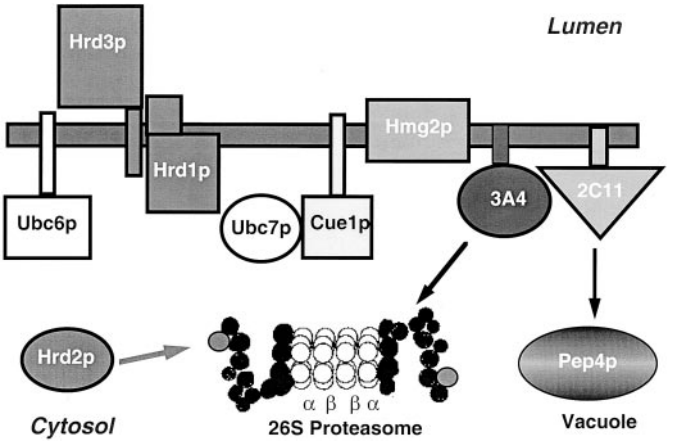
We find it noteworthy that CYP2C11 is definitely polyubiquitinated and rapidly degraded after its inactivation in isolated rat hepatocytes incubated with the suicide inactivator DDEP, although such inactivation occurs via P450 *heme N-ethylation*, with the protein left structurally unscathed (Z.-J. Song and M. A. Correia, unpublished observations). Thus, given that the in vivo half-life of the CYP2C11 heme moiety ($t_{1/2}$ = 19 ± 2 h) is comparable with that of its protein moiety ($t_{1/2}$ ≈ 20 ± 3 h) (Shiraki and Guengerich, 1984; Watkins et al., 1987), it is conceivable that the CYP2C11 normally turns over as a heme-bound protein [with its heme-thiolate ligation intact (P450) or disrupted (P420)], and this occurs via the lysosomal pathway. Indeed, because the expressed CYP2C11 exists almost entirely complexed with

heme[4] in all of the three yeast strains examined, it seems that the protein is degraded largely as a holohemoprotein rather than as an apoprotein.

On the other hand, if CYP2C11 protein is stripped of its heme moiety and the protein remains denuded due to insufficient heme for prolonged periods of time, then it may be recognized as a conformationally aberrant protein and subject to a relatively more rapid turnover via the Ub-26S proteasomal pathway, as observed in DDEP-incubated hepatocytes. This possibility is particularly intriguing, given that DDEP is an excellent short-term depletor of hepatic heme, not only because it destroys the heme of several hepatic P450s, but also because the *N*-ethylheme generated during such destruction is easily converted to an *N*-ethylporphyrin, an excellent inhibitor of heme synthesis (Ortiz de Montellano et al., 1981). Such dual DDEP-mediated heme depletion would thus prevent the structural reassembly of DDEP-inactivated, heme-stripped CYP2C11 protein and mark it for rapid disposal. Indeed, hemoproteins such as catalase and tryptophan 2,3-dioxygenase incur accelerated protein degradation after loss of their prosthetic heme and thus serve as precedents (Correia, 1991). These findings again raise the issue of whether the P450 prosthetic heme normally masks an intrinsic degron in the protein structure whose unmasking by the loss of heme targets the protein to degradation by the Ub-dependent 26S proteasome. Moreover, because heme is a well-known inhibitor of the proteasome (Etlinger and Goldberg, 1980), hepatic heme depletion may also unleash the proteasomal machinery and enhance the degradation of the heme-stripped proteins. Alternatively, the striking differences in the proteolytic targeting of DDEP-inactivated heme-stripped CYP2C11 in isolated hepatocytes may be caused by oxidative damage that occurs when the hepatocytes isolated from their normal physiological milieu become oxidatively stressed, an event that targets them to the Ub-dependent 26S proteasomal system.

Finally, we find noteworthy the relative stabilization of Sec61p and to some extent that of Sec63p in both *hrd2*-deficient and *pep4*Δ yeast strains, and this may reflect the existence of two separate cellular ER pools. Thus, it is conceivable that the Sec61p/Sec63p fractions that are stabilized in *hrd2*-deficient yeast strains reflect the pools of unassembled Sec61p/Sec63p that incur 26S proteasomal degradation (Biederer et al., 1996, 1997), whereas the corresponding fractions stabilized in the *pep4*Δ yeast strains, reflect the pools of the fully assembled Sec61p/Sec63p ER translocon. It remains to be determined whether these ER translocon proteins would also normally exhibit a biphasic turnover in common with CYP2E1.

In summary, our findings in CYP2C11-transformed *S. cerevisiae* strains reveal that the turnover of the integral native, structurally unmodified ER protein CYP2C11, unlike that of native CYP3A4, HMGR, and several substrates of the ER quality control system, is completely independent of the Ub-dependent 26S proteasomal pathway. It instead seems to involve the lysosomal (vacuolar) pathway (Scheme 1). The reasons for such differential targeting of two structurally similar proteins are not very clear, but they may entail intrinsic molecular and/or structural signals in each protein. These findings thus attest not only to the marked mechanistic diversity of ER-protein degradation, but also to its complexity. Furthermore, given that CYP2C11 can under certain circumstances also incur Ub-dependent 26S proteasomal degradation, these findings underscore the remarkable versatility of the cellular pathways for ER protein degradation.




**Scheme 1.** The versatile pathways for the degradation of ER proteins Hmg2p, CYP3A4, and CYP2C11 in *S. cerevisiae*. Adapted from Hampton and coworkers (Hampton et al., 1996; Wilhovsky et al., 2000; Gardner et al.2000; Bays et al., 2001) and Wolf and coworkers (Biederer et al., 1997; Sommer and Wolf, 1997; Plemper et al., 1999). The scheme merely depicts the relative cellular localization of the Hrd1p, Hrd2p, Hrd3p, Ubc6p, Ubc7p, and vacuolar Pep4p proteins whose relative roles in the degradation of ER-bound CYP3A4, CYP2C11, Hmg2p, Sec61p, and Sec63p were examined in our study. See the Introduction for details.

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[4] In separate experiments ($n = 3$), we ascertained that the CYP2C11 protein expressed at the tail end of the logarithmic growth phase existed entirely in the holohemoprotein form by the lack of any statistically significant differences ($p < 0.05$) between the spectrally detectable microsomal P450 content (46.7 ± 12.7 pmol/mg protein) and the corresponding immunochemically detectable CYP2C11 protein content (34.7 ± 14.6 pmol/mg protein) in RHY718, the wild-type strain from the *HRD* panel. Similar lack of significant differences between the spectrally detectable and immunochemically detectable microsomal CYP2C11 content (measured in picomoles per milligram of protein) in RHY1166, the wild-type strain from the *UBC* panel (42.3 ± 13.4 and 64.0 ± 9.84, respectively; $n = 3$), and in RHY473, the wild-type strain from the *PEP4* panel (25.3 ± 9.23 and 27.9 ± 7.58, respectively; $n = 3$), reveal that the CYP2C11 protein is largely expressed as a holohemoprotein.

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