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CYTOCHROME P450 UBIQUITINATION: Branding for the Proteolytic Slaughter?

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■ **Abstract** The hepatic cytochromes P450 (P450s) are monotopic endoplasmic reticulum (ER)-anchored hemoproteins engaged in the enzymatic oxidation of a wide variety of endo- and xenobiotics. In the course of these reactions, the enzymes generate reactive O_2 species and/or reactive metabolic products that can attack the P450 heme and/or protein moiety and structurally and functionally damage the enzyme. The in vivo conformational unraveling of such a structurally damaged P450 signals its rapid removal via the cellular sanitation system responsible for the proteolytic disposal of structurally aberrant, abnormal, and/or otherwise malformed proteins. A key player in this process is the ubiquitin (Ub)-dependent 26S proteasome system. Accordingly, the structurally deformed P450 protein is first branded for recognition and proteolytic removal by the 26S proteasome with an enzymatically incorporated polyUb tag. P450s of the 3A subfamily such as the major human liver enzyme CYP3A4 are notorious targets for this process, and they represent excellent prototypes for the understanding of integral ER protein ubiquitination. Not all the participants in hepatic CYP3A ubiquitination and subsequent proteolytic degradation have been identified. The following discussion thus addresses the various known and plausible events and/or cellular participants involved in this multienzymatic P450 ubiquitination cascade, on the basis of our current knowledge of other eukaryotic models. In addition, because the detection of ubiquitinated P450s is technically challenging, the critical importance of appropriate methodology is also discussed.

INTRODUCTION

Ubiquitination (or ubiquitylation as it is now often called) is a process in which cellular proteins are covalently modified, posttranslationally, with a single molecule (monoubiquitination) or chains (polyubiquitination) of ubiquitin (Ub) (1 and references therein). Ub is an evolutionarily highly conserved 76-residue polypeptide (8565 Da) that, as implied by its name, is ubiquitously present in all eukaryotic cells either as free species (monomers or in preformed chains) or covalently

bound to proteins. Such covalent protein decoration with Ub serves many important physiological functions. Monoubiquitination can serve as a sorting signal in endocytic vesicular transport as well as a critical regulator of transcription, replication, and DNA repair (2 and references therein). In contrast, polyubiquitination largely targets proteins for degradation via the 26S proteasome, thereby critically regulating various essential cellular processes such as cell cycle progression, antigen presentation, apoptosis and stress response, in addition to the vital function of quality control by cellular disposal of aberrant, misfolded, damaged, and/or abnormal proteins (1, 3–10). Indeed, in the latter context, high molecular mass (HMM) ubiquitinated species of certain (but not all) cytochromes P450 (P450s) have been detected in liver cells after structural damage and/or blockade of their normal physiological turnover by proteasomal inhibitors (11, 12). The HMM profile of this P450 ubiquitination and the striking temporal relationship between its detection and P450 proteasomal degradation are consistent with a role for polyubiquitination as a targeting signal in this process. Although a biological role for monoubiquitination in P450 regulation may exist, it remains obscure. Furthermore, although monoubiquitination of the various multiple P450 surface Lys residues could yield a HMM profile similar to that generated by polyubiquitination, its plausibility has been excluded by our in vitro studies with methylated Ub (MeUb) (13), the Ub analog incapable of polyubiquitination because of chemical methylation of its Lys residues. For these combined reasons, this review will focus on what is currently known about P450 polyubiquitination and its association with the proteasomal destruction of these enzymes.

THE CELLULAR POLYUBIQUITINATION MACHINERY

Protein polyubiquitination entails the covalent attachment of a chain of multiple (>4) Ub molecules most often to a Lysε-NH_2 and, albeit much less frequently, to an α-NH_2 terminus of a proteolytic substrate through the C-terminal Gly_{76} residue of the first Ub molecule in a concerted ATP-dependent process (1, 3–10; Figure 1). Three distinct classes of enzymes operate sequentially to catalyze this coupling (1, 3–10). The first is the Ub-activating enzyme (**E1**), a 100-kDa protein abundantly present in the cytosol and nuclei of eukaryotic cells. E1 contains the nucleotide binding consensus sequence Gly-X-Gly-XX-Gly (1, 4). Although comparative analysis of cDNA-derived amino acid sequences of plant, yeast, and mammalian E1s reveals five conserved Cys residues, site-directed mutagenesis studies of the wheat E1 isoform UBA1 reveal that only one of these (Cys626) is essential for its activity (14). This Cys residue is believed to reside at the E1-active site and in the presence of ATP, to be directly involved in Ub activation to a high energy ternary Ub-thioester complex (E1-S-C = OUb) via linkage to Ub-Gly_{76}. Although Ub is thus activated, it cannot be transferred directly onto the proteolytic target without the intermediacy of a second enzyme (**E2**), a member of a family of multiple Ub-conjugating enzymes (Ubcs)/Ub-carriers, as well as a third enzyme, Ub-protein

ligase (**E3**). Such sequential shuttling of the Ub-thioester from E1 to the target protein entails an initial transthiolation of an E2-Cys residue, with or without a subsequent similar transthiolation of an E3-Cys residue. This Ub relay via E2 and E3 in this process apparently insures substrate specificity by preventing the attack of random proteins by the E1 charged molecule.

E2s differ in size, usually with low molecular weights ranging between 14 and 36 kDa[1], and containing a 14–16 kDa core that is 35% conserved among family members (6–9). The remainder of the protein may contain N- and/or C-terminal extensions that confer substrate and/or E3 specificity or promote physical interactions between the three entities, often by serving as membrane anchors. Both ER-bound and soluble E2s exist for the ubiquitination of luminal and integral endoplasmic reticulum (ER) proteins such as the P450s (9, 15, 16; see below). This E2 multiplicity apparently insures functional redundancy on one hand, and substrate specificity on the other (9).

The E3 Ub-ligases, considerably more numerous than E2s, often exist as monomeric proteins or heteromeric multisubunit protein complexes. The multiplicity and structural diversity of E3s contribute to their remarkable substrate diversity and/or specificity in the recognition of proteolytic targets (9, 17–21). Three general classes of E3s are known: the HECT-E3s (Homologous to E6-AP C-terminus, the first HECT-E3 identified), CHIP-E3s (C-terminus of Hsc70 interacting protein), and the RING-finger E3s. The same E2 can apparently interact with either the HECT or the RING-finger domain of an E3 (9). HECT-E3s contain a conserved Cys-SH in their C-terminal domain for Ub-thioester relay from its cognate E2 to the target protein (9). The N terminus of some but not all HECT-E3s contains a WW domain (with 2 Trps, 20–22 residues apart and an invariant Pro within a 40-residue region) that interacts with Pro-rich sequences including those containing phosphorylated Ser/Thr residues (9, 22).

The first CHIP-E3 prototype was identified as a cochaperone of Hsc70. A typical CHIP-E3 contains a tetratricopeptide repeat (TPR) motif that interacts with both Hsc70 and Hsp90; its U-box domain exhibits E3 Ub-ligase activity. CHIP-E3s play an active role in quality control through recognition of chaperone-associated aberrant proteins that are ubiquitinated before their removal by the proteasome (17, 18). These E3s may play a similar role in the Ub-dependent proteasomal degradation of unfolded and/or misfolded P450 proteins.

The increasingly numerous RING-finger E3s, on the other hand, exhibit an interleaved or cross-braced ring pattern with eight conserved metal-binding Cys and His residues that coordinate two Zn atoms (9, 19–21). The three RING-finger motifs (RING-CH, RING-HC, and RING-H2) are distinguished by whether one or two His are the middle two conserved residues. These E3s may exist as single subunits with both substrate recognition and RING-finger E2 docking domains on the same polypeptide or as multisubunit protein scaffolds that include a small

[1]Although Ubcs are known to possess low molecular weights, a larger (582 kDa) polytopic membrane-anchored Ubc (BRUCE) has been reported (9).

RING-finger protein for E2 docking. Additional known components of these E3 scaffolds include a member of the cullin family, an F-box protein for the recognition of phosphorylated substrates (i.e., phosphorylated IκBα, Sic1, and β-catenin), and other protein subunits as intercomplex adapters (9, 23). By docking E2s, RING-finger E3s can facilitate the transfer of the activated Ub onto one of its own subunits in a regulated autoubiquitination process, or onto that of a heterologous substrate as in the case of the yeast RING-H2 finger E3 complex (Hrd1p/Hrd3p)-mediated ubiquitination of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR; 24, 25; see below). Thus, unlike HECT-E3s, RING-finger E3s mediate substrate ubiquitination by bringing the E2s in sufficiently close proximity to the protein target rather than by directly participating as an intermediary in the Ub-thioester relay.

Such E1/E2/E3-mediated coupling of Ub to one (or more) Lys residue(s) of target proteins entails channeling of the high energy of Ub-thioester hydrolysis into isopeptide bond formation. Similar linkage of the internal Lys_{48} of this Ub to the C-terminal Gly_{76} of a fresh Ub molecule results in an ordered branched chain (Ub-Ub homopolymer; PolyUb) wherein Gly_{76}-COOH of one is coupled to the Lys_{48}-εNH_2 of another Ub. It remains to be elucidated whether the formation of Ub-Ub chains involves an identical sequence of events as that involved in attaching the first Ub molecule to target substrate Lys-εNH_2 groups. Nevertheless, such polyubiquitination consisting of 4 to 20 Ub molecules in Lys_{48}-Gly_{76}-linkage not only gives the targeted protein its characteristic step-ladder appearance on SDS-PAGE/immunoblotting[2] but is also essential for targeting it to the 26S proteasome for degradation (3, 26, 27 and references therein; Figures 1 and 2). The Ub molecule has at the least seven conserved Lys residues, and Ub-Ub linkages of proteins via Lys_6, Lys_{11}, Lys_{29}, Lys_{48} and Lys_{63} have been identified (26, 27). Of these, only Lys_{48} linkages are known to function as proteolytic signals (3, 26, 27).

THE PROTEASOMAL SYSTEM FOR THE DEGRADATION OF POLYUBIQUITINATED PROTEINS

The 26S proteasome is a very large, highly complex $\approx$2000 kDa multisubunit chambered protease (27–33). Its key component is a 20S proteolytic core ($\approx$750 kDa) consisting of 28 subunits arranged in four rings (2α and 2β) each containing seven similar but functionally distinct subunits, stacked together with an outer α-ring flanking the two inner β-rings to form a pseudosymmetrical barrel with a cylindrical cavity (27, 30–33). The function and assembly of the eukaryotic 20S proteasome (previously known as the multicatalytic protease complex) is ATP-independent. Three of the β-subunits (β1, β2 and β5) harbor the protease catalytic

[2]Frequently, this is detected as a smear rather than a distinct step-ladder. However, in the case of P450s it is important to distinguish between the essential features of ubiquitination and protein aggregation.

sites with a total of six proteolytic sites/20S species (27, 30–33). Each of these three ($\beta1$, $\beta2$, and $\beta5$) sites differs in its preferential cleavage after acidic, basic, or hydrophobic residues, respectively, giving rise to the typical chymotrypsin-like, trypsin-like, peptidoglutamyl hydrolase, and caseinolytic activities of the proteasome (34). These catalytic β-subunits are unusual in exhibiting a unique catalytically active N-terminal Thr residue, a critical nucleophile in peptide bond hydrolysis, strategically lining the barrel cavity. This unusual characteristic qualifies the 20S proteasome as an amino-terminal nucleophile (NTN) hydrolase. The active site Thr residue is the target of selectively designed proteasomal inhibitors such as MG-132, lactacystin β-lactone, epoxomycin, and others (35–37).

In the eukaryotic 26S complex, either (or both) of the 20S α-ring structures is capped by a $\approx$900 kDa multisubunit complex variably termed PA700 (proteasome activator) or the 19S regulatory complex, an assembly that is ATP-dependent (27, 38–41). Thus the 26S proteasome may exist as heterooligomeric 20S barrels capped at one or both ends with this 19S complex. The 19S cap complex is composed of 17 or more subunits arranged in two structurally and functionally distinct assemblies: The base situated directly above the 20S α-ring contains six functionally distinct AAA-family ATPase or Rpt (regulatory particle ATPase) subunits and two non-ATPase Rpn (regulatory particle non-ATPase) subunits. The 19S base is topped by a lid containing eight non-ATPase Rpn subunits. In the mammalian 26S proteasome, these two structures are further linked together by the seventeenth subunit, Rpn10. High salt can dissociate the lid from the base-bound 20S proteasome, leaving a catalytic species that is capable of ATP-dependent degradation of an unfolded protein but incapable of degrading ubiquitinated substrates. This finding implies that the lid contains polyUb-recognition elements and unfoldases in addition to deubiquitinating enzymes. More recently, additional proteasome-associated proteins (including E3s) have been identified with the 19S complex, although it is unclear whether they are adventitiously bound or bona fide lid components. Together the multiple subunits of the 19S regulatory complex are responsible for the initial acceptance of the polyUb-tagged target substrates as well as the coordination of the subsequent release of this polyUb-tag with their high energy-coupled unfolding and translocation through the 20S proteolytic core to be digested (27–34, 38–41). Accordingly, the polyUb tag is initially recognized by the Rpt5 and/or Rpn10 (or any other) of the 19S base subunits, thereby tethering the substrate to the 19S subcomplex and bringing its termini or any other loosely folded domain in close proximity to one or more of the 19S base Rpt ATPase subunits for unfolding and translocation of the unfolded substrate through the 19S base pore. This event, requiring ATP hydrolysis, denatures the substrate to enable its onward movement through the juxtaposed 20S α-subunit pore into the adjacent 20S catalytic chamber where it is processively digested into short peptides that exit through the distal 20S axial pore. As the entire polyubiquitinated substrate is unfolded and strung through the 20S catalytic chamber to be proteolyzed, its polyUb tag is released intact by hydrolyses of the $UbGly_{76}$-εNH_2 substrate isopeptide bond by the 19S lid subunits Rpn11, a deubiquitinase with a Zn-metalloprotease-like domain (27, 42,

43), and Ubp6, another 19S deubiquitinase. Additional 19S subunits containing Ub-hydrolases, the cysteine proteases that disassemble the Lys_{48}-linked polyUb chain, may be subsequently recruited to regenerate Ub for fresh proteolytic cycles (27–30).

The enmeshed 20S α-subunit N termini normally clog the two gates to the adjacent 20S proteolytic β-subunit chamber, keeping its pores closed and inaccessible to peptides and unfolded proteins and therefore catalytically inactive. Thus an additional critical function of the 19S base ATPase subunits is to activate the 20S proteolytic chamber by physically lifting these α-subunit tails to open its gate to a $\approx$20 Å pore diameter that is accessible to unfolded proteins and polypeptides but not to normally folded proteins (27, 44, 45). Such restrictive gating thus protects native cellular proteins from promiscuous/indiscriminate proteasomal attack. Higher eukaryotes also contain other hybrid proteasome species, including the immunoproteasomes, that are specifically engaged in antigen processing for presentation to the immune system on major histocompatibility complex (MHC) class I molecules (35, 46–49). Because antigenic peptides from several P450 enzymes have been reported (50–53), these proteasome species are relevant to the current discussion. Unlike the above described constitutive 20S species of the 26S proteasome, that of the immunoproteasome contains interferon-γ inducible β1, β2 and β5 proteolytic subunits responsible for generating antigenic peptides (27, 49). This 20S immunoproteasome species may be capped on either or both ends by the 11S activator complex (also known as the PA28 activator, which consists of two alternating non-ATPase subunits, PA28α and PA28β, in a concentric heptameric complex of $\approx$200 kDa) (27, 49), which modulates the proteasome-catalyzed generation of antigenic peptides. The PA28 activator apparently activates the 20S proteolytic species by regulating the gating into the 20S chamber in a mechanism analogous to that of the 19S complex (27, 49).

POLYUBIQUITIN AS A 26S PROTEASOME TARGETING SIGNAL

A multitude of structurally diverse proteins incur 26S proteasomal degradation in a seemingly nonspecific process. The only structural feature that qualified 26S proteasomal substrates [with the exception of ornithine decarboxylase (54, 55) and p21Cip1 (56)] exhibit in common is a Lys_{48}-Gly_{76}-linked polyUb chain attached to an ε-NH_2 group of a Lys-residue (and less frequently to an α-NH_2) of the protein target. Although preferential Lys residues exist for ubiquitination, in their absence alternative Lys residues can substitute in some instances. Such protein tagging as discussed above, albeit insufficient for degradation, is essential for its recognition as a proteasomal substrate. And as long as this polyUb tag remains both viable as a recognition signal and latched firmly onto the 19S cap to induce protein unfolding and translocation into the proteolytic chamber, the ubiquitinated substrate, whether monomeric or a subunit of a heteromeric complex, is irrevocably committed to 26S

proteasomal degradation (Figure 2). Any specific substrate sorting for proteolysis thus has to occur before the protein is polyubiquitinated.

Indeed, such specific substrate sorting does occur and E3s apparently play a critical role in the selection of a substrate for Ub-dependent 26S proteasomal degradation through the recognition of specific substrate-based structural determinants that partly or fully constitute destruction signals called degrons (9, 27). Such critical structural determinants of substrate recognition by E3s may be intrinsic to the protein's primary sequence or acquired through posttranslational processing. Degrons encoded in a short discrete sequence include the Deg1 sequence of the yeast *MATα2* transcriptional regulator, the destruction box of mitotic cyclins, the degradation motif of IκB proteins, the stability regulating region of cMOS, specific phosphorylatable Ser/Thr residues, PEST sequences, Pro-rich domains, and some N-terminal residues of target proteins (9, 27; reviewed in Reference 57). On the other hand, instead of a discrete modular degron, the structural information for Ub-dependent 26S proteasomal degradation may be distributed over a considerably large protein domain, as in the case of the entire N-terminal 523-residue-long transmembrane domain of HMGR (58, 59) as well as the IκBα N-terminal phosphorylatable (Ser_{32}/Ser_{36}) and ubiquitinatable (Lys_{21}/Lys_{22}) and C-terminal PEST domains (60–62). Although certain P450s are indeed phosphorylated (13, 63–69) and/or ubiquitinated (13, 69) before their proteolytic degradation, it remains to be determined whether they similarly harbor any intrinsic modular or distributed degrons that are either normally accessible or unmasked for this event.

HEPATIC P450 UBIQUITINATION

The first clue that P450s might be subject to Ub-dependent 26S proteasomal degradation was provided by two independent 1992 reports (11, 70). In the first, immunoblotting analyses of liver microsomes revealed the strikingly enhanced formation of HMM Ub-conjugated liver microsomal proteins within 30 min of CCl_4 administration to mice compared with that seen in vehicle-treated controls (70). This HMM microsomal ubiquitination profile was only slightly subdued at 1 h after CCl_4, but markedly attenuated after 5 h. This profile correlated well with the time-dependent immunochemically and functionally detectable proteolytic loss of microsomal CYP2E1, a known target of CCl_4-mediated inactivation. CCl_4 is known to inactivate CYP2E1 in a mechanism-based process that results in heme fragmentation and irreversible modification of the P450 protein by the ensuing heme fragments (71), presumably at the active site. The close temporal association of the two profiles led to the proposal that the observed HMM ubiquitinated microsomal protein included CYP2E1 species, although no HMM anti-CYP2E1 immunoreactivity was concomitantly detected (70). The failure to detect any immunoreactive HMM CYP2E1 species was rationalized by the low abundance of HMM microsomal CYP2E1 species and/or plausible masking of its epitopes by Ub-conjugation (see below). It is relevant to note that the parallel microsomal CYP2E1 inactivation by the pan-P450 mechanism-based inactivator, 1-aminobenzotriazole (ABT),

yielded no comparable proteolytic loss of the enzyme nor a similar HMM ubiquitination profile (70). ABT inactivates most P450s via heme-N-arylation rather than heme-modification of the protein (72). However, although ABT rapidly and effectively abolished CYP2E1 function, minimal CYP2E1 proteolysis was observed along with relatively minor ubiquitination of the microsomal protein detectable only after 9 h of ABT treatment (70). Thus, despite marked P450 functional loss, the protein moiety apparently escapes unscathed and remains relatively stable.

The second report documented the time-dependent ubiquitination and proteolytic loss of rat liver microsomal CYPs 3A after their mechanism-based inactivation by 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP), a 4-ethyl analog of the calcium channel antagonist nitrendipine (11, 73, 74). DDEP-induced CYP3A inactivation also results in heme-modification of the protein that is accompanied within 30 min by ubiquitination of the microsomal protein and immunochemically detectable loss of these microsomal enzymes (11, 73, 74). This ubiquitination is, as expected, enhanced by the coadministration of hemin, a known 26S proteasome inhibitor (75–77; see below). More importantly, when the microsomal CYPs 3A were first immunoprecipitated with goat anti-CYP3A23 IgGs and then immunoblotted with rabbit anti-Ub IgGs, they exhibited the characteristic step-ladder ubiquitination profile discussed earlier (11; Figure 3*a*). This finding unequivocally established that the DDEP-inactivated CYPs 3A were indeed ubiquitinated. This DDEP-induced ubiquitination of liver microsomal CYPs 3A in intact rats could be reproduced in DDEP-incubations of freshly isolated hepatocytes obtained from dexamethasone (DEX)-pretreated rats (12). This system provided a convenient experimental model for the definitive mechanistic characterization of the proteolytic process. Accordingly, CYP3A immunoprecipitation analyses revealed that incubation of these freshly isolated hepatocytes with DDEP resulted in the ubiquitination of CYPs 3A within 15 min of their inactivation, an event that preceded the onset of their proteolytic degradation detectable at 30 min (12). Inclusion of the proteasomal inhibitors aclarubicin or MG-132 in these incubations, while blocking the DDEP-induced immunochemically detectable loss of microsomal CYPs 3A, also intensified the CYP3A ubiquitination profile (12). These findings in freshly isolated rat hepatocytes thus unequivocally established that DDEP-inactivated, heme-modified CYPs 3A undergo Ub-dependent 26S proteasomal degradation (12).

Both ubiquitination and 26S proteasomal degradation of heme-modified CYPs 3A can also be documented in in vitro reconstituted systems (13) containing purified recombinant CYP3A4, the major human liver CYP3A ortholog. For this purpose, ^{35}S-labeled CYP3A4 either native or heme-modified by inactivation with cumene hydroperoxide (CuOOH) and Fraction II (a rat liver cytosolic subfraction containing the requisite soluble E1, E2 and E3 enzymes and the 26S proteasome) were incubated in the presence of Ub, an ATP-generating system, protease inhibitors (to block the ubiquitous lysosomal protease contaminants), $MgCl_2$, and Ub-aldehyde (Ubal). The inclusion of Ubal, an inhibitor of Ub-hydrolases and

isopeptidases, in these incubations is essential for blocking protein deubiquitination so that Ub-conjugation of a substrate can be detected. In the absence of other proteins, thermally sensitive CYP3A4 tends to aggregate on incubation at 37°C, thereby generating undesirable and confounding cross-linking artifacts (see below). To preclude such incubation-induced aggregation artifacts, liver microsomal membranes from female rats that are devoid of appreciable CYP3A content and exhaustively washed free of luminally trapped cytosolic ubiquitinating and proteolytic enzyme contaminants were included as a membrane platform for CYP3A4.[3] Parallel immunoblotting analyses of these incubates with either anti-CYP3A or anti-Ub IgGs revealed that only the heme-modified but not the native CYP3A4 exhibited a time-dependent ubiquitination profile which was enhanced by the inclusion of the proteasome inhibitor, Z-IE(OtBu)ALCHO (PSI) (78). No CYP3A4 ubiquitination profile was detected if Fraction II was omitted from the incubations (13). Moreover, no similar profile was observed when Ub was replaced by MeUb (the methylated Ub-analog incapable of any Lys_{48}-Gly_{76} polyubiquitination linkages; see above) in the incubation (13). If appreciable CYP3A4 aggregation were to have occurred, it should have been detected in the incubations with MeUb. The absolute dependence of the observed CYP3A4 ubiquitination profile on both Fraction II and Ub convincingly attests to its authenticity, while excluding the recently raised possibility that this finding represents a CYP3A4 aggregation artifact (79, 80).

Two native rat liver P450s, CYP2B1 and CYP2C11, that have relatively long half-lives and reportedly are degraded by the lysosomal pathway in vivo, are also subject to Ub-dependent 26S proteasomal degradation when suicidally inactivated (13, 57, 81). Accordingly, in an in vitro reconstituted system similar to the one described above, CuOOH-inactivated heme-modified CYP2B1 was shown to be ubiquitinated and degraded by the 26S proteasome-species (13). On the other hand, DDEP incubation of freshly isolated hepatocytes from untreated male rats also caused a time-dependent structural and functional inactivation of CYP2C11 that is associated with CYP2C11 protein ubiquitination and proteolytic loss (Z.-J. Song & M.A. Correia, unpublished observations; 81). Although such DDEP-induced CYP2C11 inactivation is largely due to its prosthetic heme destruction to an N-ethylporphyrin, with little or no heme modification of the protein, no structural or functional restoration of the enzyme was observed when hemin was included in the incubations (81). Instead, as revealed by CYP2C11 immunoprecipitation analyses, inclusion of hemin in the DDEP-incubations resulted in an accumulation of ubiquitinated CYP2C11 species, consistent with hemin blockade of proteasomal function at a step beyond protein ubiquitination (Figure 3*e*). Unlike the recent findings in primary hepatocytes in culture (79), incubation of

[3]In retrospect, this strategy for preventing CYP3A4 aggregation was fortuitous, given that ERAD substrates such as CYP3A4 apparently require integral and ER-associated enzyme components for their ubiquitination. The inclusion of ER membranes inadvertently provided the ER ubiquitination components later found to be essential (16, 113).

hemin alone with otherwise untreated hepatocytes yielded no detectable P450 aggregation/cross-linking HMM artifacts (Figure 3*e*). CYP2C11 inactivation by DDEP is mechanistically similar to that of CYP2E1 by ABT in that the heme moiety of both isoforms is N-modified by the inactivators. Yet, albeit intriguing, it is unclear why the heme-stripped CYP2C11 is a target for ubiquitination whereas the heme-stripped CYP2E1 is not. It is conceivable that this CYP2C11 susceptibility to the ubiquitination machinery is determined by discrete modular degrons in its protein sequence that are unmasked on prosthetic heme destruction.

Finally, CYP2E1 Ub-conjugation has been documented in two in vitro systems (82, 83), which to our knowledge, are the only reported sightings of ubiquitinated CYP2E1. The first used CYP2E1-enriched rat liver microsomes incubated at 37°C for 1 h with untreated rat liver cytosol as the ubiquitination/proteolytic system supplemented with leupeptin, aprotinin, α_2-macroglobulin, $MgCl_2$, ATP, NADPH, and with or without CCl_4 (20 mM) as the CYP2E1 suicide inactivator. Microsomes were reisolated and probed by immunoblotting with either anti-CYP2E1 or anti-Ub antibodies. These findings revealed that immunoreactive HMM CYP2E1 species and Ub-conjugates were detected, particularly after CCl_4-inactivation of CYP2E1 and to a lesser extent in its absence (82). However, several methodological concerns render these findings somewhat unconvincing. The first is that no attempt was made to isolate CYP2E1 by immunoprecipitation for verification that it was truly ubiquitinated. As previously indicated (70), the HMM ubiquitinated species detected in microsomal immunoblots would reflect Ub-conjugates of myriad microsomal proteins including CYP2E1. Second, because CCl_4 is a notorious inducer of lipid peroxidation and no attempt was made to block NADPH/CCl_4-induced microsomal lipid peroxidation, P450 cross-linking with itself and/or other Ub-conjugated microsomal proteins could also account for the HMM protein species as previously reported (84), and recently confirmed with CYPs 3A (80). The latter possibility is particularly likely given that the functionally robust hepatic deubiquitinases were not blocked in these studies (82), a sine qua non for detectable hepatic protein ubiquitination. Third, the principal author was unable to confirm these findings in a subsequent report (85), invoking instead a major role for the Ub-independent 20S proteasomal species in CYP2E1 degradation.

The second report describes ^{35}S-CYP2E1 ubiquitination after its translation from in vitro transcribed RNA in a rabbit cell-free reticulocyte lysate translation/ubiquitination system (83). This CYP2E1 ubiquitination was apparently enhanced by the inclusion of the proteasome inhibitor MG-132 in this system. Through immunoinhibition and modeling studies, this ubiquitination was postulated to occur on Lys_{317} and Lys_{324} residues in the putative cytosol-exposed CYP2E1 J-helix-J′-loop domain (83). No comparable CYP2E1 ubiquitination was observed when a wheat germ lysate translation system that lacks the ubiquitination machinery was employed. The in vitro ubiquitination studies of newly translated CYP2E1 reveal that the protein is ubiquitinated on two of its cytosol-exposed Lys residues (83; see above). However, even though a large protein domain is presumably exposed to the cytosol, only minimal ubiquitination of native hepatic CYPs 3A is normally

detected in vivo, even under conditions optimized for its maximal detection (Figure 3*a*,*c*; see below). This ubiquitination is considerably augmented after the protein is structurally unraveled by catalytic insults such as futile oxidative cycling and/or chemically induced suicide inactivation. Such structural damage may expose normally concealed Lys residues and/or other degron components such as phosphorylatable residue PEST sequences and other targetable domains to the ubiquitinating enzymes. Together, the above findings attest to the indisputable fact that when inactivated, misfolded, or otherwise structurally deformed, certain hepatic P450s incur ubiquitination and/or 26S proteasomal degradation. But are the native CYPs 3A also ubiquitinated in the course of their physiological turnover?

NATIVE P450 UBIQUITINATION: THE CELLULAR LOCUS, PARTICIPANTS, AND LESSONS FROM THE YEAST

Hepatic P450s are monotopic proteins, N-terminally anchored to the ER with their catalytic domain facing the cytosol wherein a substantial cellular inventory of the ubiquitination machinery and/or the 26S proteasome are located. As ER residents and documented substrates of Ub-dependent 26S proteasomal degradation, CYPs 3A qualify as bona fide models for the mechanistic characterization of hepatic ER-associated degradation (ERAD). In analogy to the ERAD of other cellular proteins (86–88), ER-associated P450 ubiquitination in hepatocytes most likely involves hepatic ER-associated Ub-conjugating E2 enzymes and E3 Ub-ligases, whose specific identities remain to be divulged. However, the dilemma was in identifying a suitable experimental model wherein this physiological process could be mechanistically dissected without confounding inherent artifacts. As discussed previously (57), P450s are not very stable and turn over rapidly in cell-lines or when hepatocytes are cultured (57, 89–92). This led to the serious consideration of yeast as a model.

Until very recently, most of our knowledge of the ERAD of integral and luminal proteins (86–88) was derived from genetic analyses of the yeast *Saccharomyces cerevisiae*. Studies of the polytopic ER protein Hmg2p (the sterol-regulated yeast isoform of HMGR, the rate-limiting enzyme in sterol synthesis) and of CPY* (a misfolded carboxypeptidase mutant retained in the ER-lumen) have uncovered *HRD* (HMGR Degradation) and *DER* (Degradation in ER) genes, respectively (86–88). This HRD/DER machinery includes (*a*) the ER-associated Ub-conjugating enzymes (Ubc1p, Ubc6p, and Ubc7p). Ubc6p and Ubc7p are key enzymes in the ERAD of luminal and membrane-bound proteins in yeast proteins (86–88, 93–98). Ubc6p is an integral C-terminally anchored ER-protein with its N-terminal catalytic domain exposed to the cytosol, whereas Ubc7p is a cytosolic protein that requires an integral membrane-anchored partner Cue1p for its catalytic participation in ERAD. Both Ubc6p and Ubc7p/Cue1p are required for the ubiquitination of certain polytopic ER-proteins (15, 93–98) except HMGR which requires only

Ubc7p/Cue1p but not Ubc6p (93, 97, 98). The HRD/DER machinery also includes (*b*) Hrd2p, a 19S protein subunit that is a functionally indispensable component of the 26S proteasome (98), and (*c*) Hrd1p/Hrd3p complex, the ER-associated Ub-ligase (E3) composed of Hrd1p/Der3p and its partner, Hrd3p. Hrd1p is an integral ER-membrane protein with two distinct domains: a multitransmembrane-spanning N-terminal hydrophobic region and a cytosolic C-terminal hydrophilic RING-H2 motif (24, 25, 99, 100) that binds Ubc1p or Ubc7p. Hrd3p is an ER glycoprotein with a single C-terminal membrane-anchor and a large N-terminal domain in the ER-lumen. Hrd3p is found to stabilize Hrd1p in the ER membrane (24). The Hrd1p/Hrd3p complex catalyzes the Ubc7p-dependent ubiquitination of target substrates such as Hmg2p and CPY* (24, 99–101). The HRD/DER machinery also includes Cdc48p (p97, an AAA ATPase required for cellular processes such as cell division, protein degradation, and ER membrane fusion), Npl4/Hrd4p, an ER-specific adapter of undefined function, and Ufd1p, a Cdc48p protein adapter for polyUb chain recognition and/or Cdc48p-association with Hrd4p (102–104). The Cdc48p-Ufd1p-Hrd4p complex is apparently involved in the recognition of polyubiquitinated luminal and integral ER proteins, their dislocation from the ER, and their subsequent delivery to the 26S proteasome. Mammalian homologs of the yeast HRD/DER machinery such as Ubc6p, Ubc7p, Cue1p, Hrd2p, Hrd1p Ufd1p, Hrd4p and Cdc48p have been recently documented (102–112), indicating that ERAD is evolutionarily a highly conserved process.

Because of this high homology between yeast and mammalian ubiquitination enzymes and the availability of validated genetic *S. cerevisiae* strains with defined defects in the ubiquitination and proteasomal degradation of several integral ER proteins including Hmg2p, the yeast model was used to identify and characterize the enzymes participating in the ubiquitination of CYP3A4, the dominant human liver P450 (113, 114). To identify the Ubc involved in CYP3A4 ubiquitination, isogenic wild-type (*wt*) yeast strains and strains deficient in Ubc6p, Ubc7p, or in both Ubc6p and Ubc7p were transformed with the CYP3A4 expression vector pAAH5/NF25 or the control vector (113). At the early stages of logarithmic cell growth, CYP3A4 was equivalently expressed in all four strains, indicating their comparable transcriptional and translational efficiencies (113). At the later stages of culture, CYP3A4 was greatly stabilized only in mutants deficient in Ubc7p and Ubc6p/Ubc7p but not in Ubc6p alone, thereby revealing the relative importance of Ubc7p-dependent ubiquitination in the ERAD of this native integral protein. Thus the monotopic CYP3A4 and the polytopic Hmg2p are alike in that they require Ubc7p-dependent ubiquitination for their ERAD. Presumably, such Ubc7p-mediated CYP3A4 ubiquitination also requires the ER-protein adapter Cue1p.

To determine whether the RING-H2 Hrd1p/Hrd3p Ub-ligase complex and Hrd2p involved in Hmg2p Ub-dependent proteasomal degradation were similarly involved in that of CYP3A4, isogenic *wt* and mutant *hrd1*Δ, *hrd2-1*, and *hrd3*Δ *S. cerevisiae* strains were transformed as discussed above with a CYP3A4 expression plasmid and corresponding vector control. CYP3A4 protein was equivalently expressed in all four yeast strains during the early logarithmic growth phase, but at the

later stationary growth stage of culture, CYP3A4 protein was comparably reduced in *wt* and *hrd1*-deficient yeast, consistent with its degradation predominating over its de novo synthesis after transient expression of both strains (113). In contrast, consistent with the role of 26S proteasome in CYP3A4 degradation, the microsomal CYP3A4 protein content was significantly stabilized in *hrd2*-deficient yeast. These findings thus reveal that in yeast, the RING-H2 finger Ub-ligase Hrd1p is not required for CYP3A4 Ub-dependent proteasomal degradation in contrast to that of Hmg2p (113). It is unclear whether the lesser, albeit statistically significant, CYP3A4 protein stabilization observed in *hrd3*-deficient yeast reflects the interaction of Hrd3p with an Ub-ligase partner other than Hrd1p. The yeast Ub-ligase required for CYP3A4 ubiquitination currently remains to be identified. Any of the several ERAD-associated E3 ligases such as the ER-localized yeast RING-CH Doa-10 or HECT-like Rsp5p (115, 116) remain plausible E3 candidates in CYP3A4 ubiquitination. Similarly, the recently identified human Hrd1p homolog HRD1 (110) or its related mammalian E3 homolog gp78/AMFR (autocrine motility factor receptor; 111) could be involved in CYP3A4 ubiquitination in the human liver. Because all these E3 ligases duly engage Ubc7p or its human counterpart UBC7 in their CYP3A4 ubiquitination reactions, their E3 candidacy is plausible.

It is noteworthy, however, that the rapid disposal of human liver CYP3A4 via the Ub-dependent 26S proteasomal degradation in *S. cerevisiae* is not because it is an alien protein. Corresponding expression of other mammalian P450s (rat liver CYPs 2B1 and 2C11) in these same yeast strains (114, 117) results in their vacuolar lysosomal degradation rather than proteasomal degradation. The latter findings are not only consistent with similar observations in intact rats (57 and references therein), but also confirm the validity of the yeast model for mammalian P450 turnover analyses.

P450 UBIQUITINATION: THE TRAJECTORY TO PROTEASOMAL DEGRADATION

CYP3A immunoprecipitation analyses of ER (microsomal) and cytosolic subfractions over the time course of their DDEP inactivation in isolated hepatocytes revealed that the ^{35}S-labeled CYPs 3A initially present in the ER were translocated into the cytosol as their proteasomal degradation progressed. Treatment with the proteasomal inhibitor aclarubicin resulted not only in decreased CYP3A proteolysis but also in the impaired translocation of the ubiquitinated ER-bound CYP3A species into the cytosol (12). Consequently, ubiquitinated CYP3A species accumulated in the ER, consistent with aclarubicin blockade at a step beyond the ubiquitination of these enzymes. These findings thus indicate that the ERAD of DDEP-inactivated CYPs 3A entails their initial polyubiquitination while still incorporated in the ER, with subsequent dislocation from the ER membrane and translocation into the cytosol (12). These results also strongly suggest that the

cytosolic rather than the ER-associated 26S proteasome species is involved in CYP3A ERAD. Furthermore, given that the inhibition of the proteasome blocked CYP3A dislocation from the ER, it appears that a functional proteasome species is required for this event. How exactly the ubiquitinated P450 is delivered from the ER to the cytosolic 26S proteasome is an issue that remains to be elucidated.

The characterization of the export into the cytosol of other mammalian ERAD substrates such as the luminal unassembled heavy chain of secretory immunoglobulin M (IgM) and the integral MHC class I heavy chains provides excellent paradigms (105–109, 112). From these models several instructive details can be gleaned: (*a*) polyubiquitination (with intrinsic Lys_{48}-linkage) rather than monoubiquitination is required but insufficient for degradation; (*b*) subsequent to ubiquitination, ATP γ-phosphate hydrolysis is required for the release of the ER-bound substrate into the cytosol; (*c*) the polyUb chain serves as a recognition signal rather than as a ratcheting molecule that drives the protein out of the ER; (*d*) proteasome function is not required for ER protein ubiquitination but may be required for protein dislocation; (*e*) the chaperone Bip (Hsp70) may facilitate this process; and most importantly, (*f*) a downstream component, the mammalian cytosolic ERAD chaperone p97-Ufd1-Npl4 complex (the equivalent of yeast Cdc48p-Ufd1p-Hrd4p), is involved in the polyUb recognition step and ATP-hydrolysis. Apparently, the AAA ATPase p97 is responsible for the ATP hydrolysis, whereas the N-terminal Ufd1 Ub-binding domain (rather than the Npl4-Ub binding domain) that specifically recognizes $UbLys_{48}$-linkages is responsible for the Ub-recognition (109, 112). Although the exact role of p97 remains controversial, its participation in the ER to cytosol translocation of these ERAD substrates is indisputable (109, 112). Similarly, it is unclear whether ERAD involves just the 26S proteasomal subpopulation recruited to the ER by p97 or its entire cytosolic pool. A notable difference between the translocation of these model ER proteins and that of CYPs 3A is that very little (if any) of the P450 protein domain is luminally oriented, whereas only a small $\approx$27-residue N-terminal tail is ER-membrane bound, with most of the protein already facing the cytosol and poised for ubiquitination and/or proteasomal degradation. Thus it remains to be determined whether the protein is translocated intact or dislocated from its N-terminal anchor before it is ubiquitinated and/or delivered to the proteasome.

P450s: THE DEFIANT ONES

CYP2E1 normally exhibits a biphasic turnover, with the rapid turnover species undergoing degradation that is inhibited by proteasome inhibitors (82, 85, 91, 92, 118, 119) and the long turnover species being degraded by an autophagic-lysosomal pathway, sensitive to lysosomal inhibitors (120, 121; reviewed in Reference 57). However, the in vitro CYP2E1 ubiquitination reports discussed above notwithstanding, no HMM ubiquitinated CYP2E1 species could be immunoprecipitated from hepatocytes or HepG, Tc-HeLa, and Fr-8a2 cell lines (92). Indeed, studies on stably expressed CYP2E1 in E36ts20 (a cell line with a temperature-sensitive

Ub-activating E1 enzyme) revealed no significant stabilization of CYP2E1 turnover at the nonpermissive temperature when compared with that in the corresponding parental E36 cells (92). In contrast, as expected, the ubiquitination of two known substrates (oxidized RNase and c-Jun) was apparently impaired at the nonpermissive temperature in the E36ts20 but not parental cells (92). Furthermore, mutation of CYP2E1 Lys_{317}, Lys_{324}, or of both these residues (reportedly ubiquitinated in vitro; 83) had no appreciable effect on CYP2E1 turnover in COS-1 cells (92). Together these findings confirmed that CYP2E1 turnover, irrespective of the cell type, is Ub-independent. Thus, although CYPs 3A and 2E1 share many common characteristics such as a propensity for futile oxidative cycling, relatively short in vivo half-lives, and induction via substrate-mediated stabilization, they apparently differ in their susceptibility to Ub-dependent 26S proteasome degradation. However, given its relative ER abundance, it is conceivable that the fraction of ubiquitinated CYP2E1 is too miniscule for detection. Whatever the reason for its recalcitrance to ubiquitination, the fact that CYP2E1 turnover was unequivocally inhibited by proteasome inhibitors (85, 92, 119), suggests that the 20S rather than the 26S proteasome species is involved. These findings raise the provocative issue of how, in the absence of a polyUb targeting signal, this ER-anchored enzyme is dislocated, shuttled to the 20S proteasome, unfolded, and threaded through its catalytic barrel. The requirement for ATP and the chaperone Hsp90 (122) and the superiority of cytosol versus the purified 20S proteasome species may reflect the involvement of yet to be identified cytosolic ATP-dependent dislocases and/or unfoldases. It is also plausible that the ER-associated proteasome subpopulation is responsible for the observed CYP2E1 degradation.

P450 UBIQUITINATION: A METHODOLOGICAL POSTSCRIPTUM

The detection of protein ubiquitination is technically tricky and requires careful methodological approaches. Protein ubiquitination is a dynamic process, with polyUb chains being put on the protein and taken off both as the ubiquitinated protein is proteasomally degraded and/or the polyUb chains are disassembled by the ubiquitous and avid deubiquitinases (1, 123). Thus only a small fraction of the ubiquitinated protein can be captured at any given time. Reliable capture of a detectable fraction of ubiquitinated proteins therefore often requires inhibition not only of the proteasome by specific inhibitors but also of the deubiquitinases by inhibitors such as Ubal and N-ethylmaleimide (NEM) (1, 123–125). This is particularly true of the detection of hepatic ubiquitinated P450 proteins. Not only are the liver deubiquitinases abundant and highly robust (12, 70, 124), but the subfractionation process to isolate the microsomal P450s also results in the release of undesirable lysosomal proteases that can inactivate the essential ubiquitinating enzymes as well as degrade P450 proteins (126). Thus maximal trapping of the ubiquitinated P450s from the liver requires homogenization buffers supplemented with general protease

inhibitors and NEM. Furthermore, in vitro ubiquitination reactions with cytosolic Fraction II require the presence of lysosomal protease inhibitors and Ubal[4]. Even after all these precautions, it is often difficult to detect significant P450 ubiquitination by immunoblotting analyses, unless the immunoblots are hydrated by autoclaving (127). The use of ^{125}I-labeled Ub and autoradiography/PhosphorImager analyses can circumvent these problems and considerably improve the detection sensitivity (128). Even so, definitive detection of ubiquitinated P450s requires that the proteins be immunoprecipitated from the liver microsomes or ubiquitination reactions. This is particularly critical when P450 ubiquitination is examined in vitro, because preformed but unattached polyUb chains in the reaction mixture can also yield the HMM profile characteristic of a ubiquitinated protein.

Immunoprecipitation from liver microsomes is also essential because liver P450s, particularly CYPs 3A and CYP2E1, are known to be highly sensitive to structural insults derived from futile oxidative cycling, lipid peroxidation, storage, and thermal changes, all of which can result in aggregation and/or cross-linking of the P450s intermolecularly and/or with other microsomal proteins (80, 84, 129–132). Thus when liver microsomes are used as the P450 source, such protein aggregation and/or cross-linking artifacts can greatly confound the determination of whether a P450 is ubiquitinated and/or degraded[5] by immunoblotting analyses and lead to flawed conclusions. However, as discussed below, the P450 aggregation and ubiquitination profiles can be readily distinguished on careful inspection.

Figures 3 and 4 illustrate some of the critical methodological issues, such as the importance of using freshly prepared liver microsomes, optimal storage conditions, and immunoprecipitation procedures, for maximal detection of ubiquitinated P450s by immunoblotting analyses, with CYP3A as an example. For this purpose, the hepatic microsomal CYP3A content was enriched by DEX-pretreatment of rats, followed by DDEP administration to inactivate the P450s. Liver microsomes obtained from DEX- or DEX/DDEP-treated rats were immunoprecipitated with polyclonal goat antirat CYP3A23 IgGs (Figure 3*a*). Note the time-dependent rat liver CYP3A ubiquitination maximally detected at 60 min after DDEP-treatment (Figure 3*a*). The corresponding Western CYP3A immunoblots of these liver microsomes, deliberately overexposed to enhanced chemiluminescence (ECL) detection, are shown in Figure 3*b*. These immunoblots reveal time-dependent DDEP-induced CYP3A loss (at ≈55 kDa), without any concurrently detected CYP3A aggregates (dimers, trimers, and/or oligomers), even on ECL overexposure (Figure 3*b*). The relative importance of NEM inclusion during liver homogenization for an appreciable intensification of microsomal CYP3A ubiquitination is shown in Figure 3*c*. Because the goat IgGs used for CYP3A immunoprecipitation were fractionated

[4]NEM would inactivate the sulfhydryl groups of several proteins including the ubiquitinating enzymes, and should therefore be used only at the termination of the ubiquitination reactions.
[5]The upward migration of CYP3A due to the formation of dimers, trimers, and/or oligomers effectively reduces the levels of the parent proteins detected at ≈55 kDa, thereby leading to the erroneous conclusion that P450 is lost because of protein degradation.

from immunized goat serum and thus were not fully purified, the corresponding immunoblot background reflecting the presence of serum contaminants is also shown with a mock immunoprecipitate from mixtures without liver microsomes (Figure 3*c*; last lane). Densitometric assessment of CYP3A4 ubiquitination requires that this background be subtracted from the corresponding profiles in the other four lanes. Figure 3*d* illustrates the absolute need for autoclaving in the immunoblotting analyses of P450 ubiquitination, particularly if neither the Ub nor P450 molecules are radiolabeled and thus undetectable by autoradiography or PhosphoImager analyses. Autoclaving of the electroblotted membranes is found to improve the immunodetection of polyUb epitopes by rehydration of the denatured proteins (127).

The source of the confounding artifacts often encountered during in vitro P450 ubiquitination assays are illustrated in Figure 4. Freshly prepared liver microsomes from DEX-pretreated rats (stored as pellets at -80°C for ≤ 1 wk) were used for CYP3A immunoblotting analyses before or after incubation at 37°C for 2 h, under conditions identical to those previously detailed (129), except that $CaCl_2$, $ZnCl_2$ or $MgCl_2$ were individually included instead of the previously used Ca^{+2}/Zn^{+2} combination. Note that in the nonincubated (0 h) microsomes, no CYP3A aggregation was detected even after sample overload (Figure 4*a*). Incubation of these microsomes at 37°C for 2 h in the presence of $CaCl_2$, $ZnCl_2$, or $MgCl_2$ only minimally increased the detection of CYP3A dimers and trimers, even after sample overload (Figure 4*a*). Figure 4*b* depicts the corresponding protein ubiquitination pattern of the nonincubated (0 h) microsomes. This profile was clearly enhanced by the inclusion of NEM during homogenization of the liver (Figure 4*b*).

On the other hand, immunoblotting analyses of liver microsomes stored as pellets at -80°C for longer periods ($\geq$1 year) clearly documented the formation of CYP3A dimers and trimers even without incubation (Figure 4*b*). Furthermore, immunoblotting analyses of these stored microsomes after incubation at 37°C for 2 h as described (Figure 4*a*) clearly revealed the presence of CYP3A oligomers/aggregates at the stacking/running gel interphase (Figure 4*c*; marked with an asterisk) in addition to CYP3A dimers and trimers and irrespective of the cation present. Corresponding immunoblotting analyses with anti-Ub IgGs of incubations depicted in Figure 4*c* are shown in Figure 4*d*. Note the salient differences in this profile and that seen in Figures 3*a* and 3*c*, particularly at the stacking/running gel interphase (marked with an asterisk). Additional storage of the nonincubated microsomal suspensions for just a week at -20°C resulted in the dramatic CYP3A aggregation, which was further intensified by incubation at 37°C for 2 h (Figure 4*e*). Only slightly lesser aggregation was detected if instead these suspensions were stored at -80°C for a week (Figure 4*e*).

Collectively, these findings clearly indicate that maximal detection of liver P450 ubiquitination requires (*a*) addition of NEM during liver homogenization; (*b*) minimal (<2 wk) storage of microsomal pellets at -80°C; (*c*) immunoprecipitation of the P450 under scrutiny; and (*d*) autoclaving of the electroblotted membranes for maximal immunochemical detection. Artifacts such as those depicted in

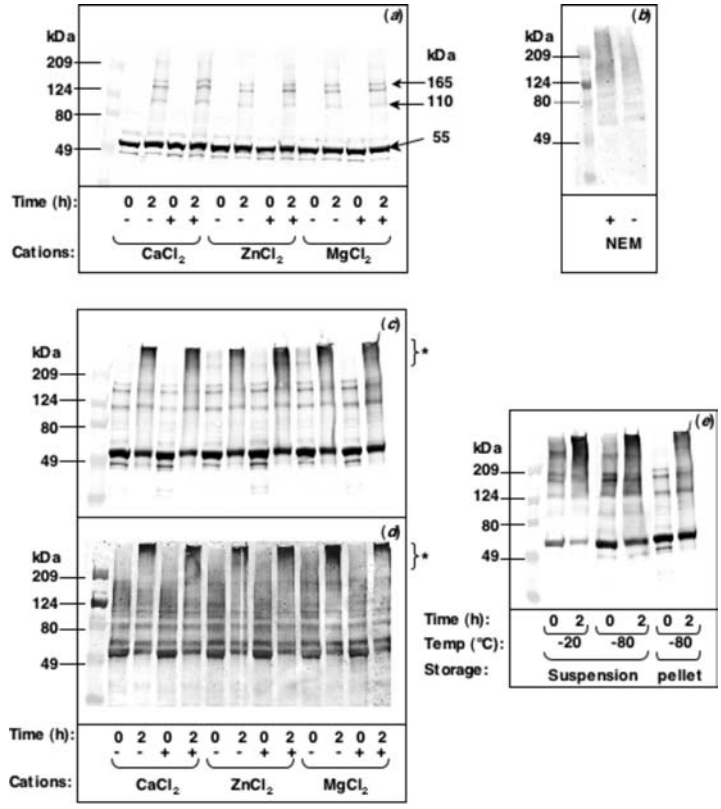

Figure 4 CYP3A aggregation: Effects of microsomal storage, incubation, NEM and/or divalent cations. (*a*) Freshly prepared liver microsomes (75 μg) were incubated at 37°C for 0 or 2 h, in the presence of 25 mM sucrose, 0.154 mM KCl, 1 μM Ub, and 2 mM $CaCl_2$, 3 μM $ZnCl_2$, or 10 mM $MgCl_2$ in 50 mM Tris.HCl buffer, pH 7.5. Reactions were terminated with an equal volume of sample loading buffer [50 μl; corrected version (129)]. An aliquot (7.65 μg protein) was loaded onto a 4–20% gradient Tris-HCl ready gel (BioRad) for CYP3A immunoblotting analyses using a primary goat antirat CYP3A23 antibody (1:10,000, v/v) overnight, followed by a secondary swine antigoat AP-conjugated antibody (1:3000, v/v) for 1 h followed by color development. (*b*) Nonincubated freshly prepared microsomes (15.3 μg) from DEX-pretreated rat livers homogenized with (+) or without (−) 5 mM NEM were subjected to immunoblotting analyses against a goat anti-Ub antibody exactly as described (Figure 3*a*). (*c*) Liver microsomes from DEX-pretreated rat livers stored at −80°C as pellets overlaid with 10% glycerol/0.1M phosphate buffer, pH 7.4, for ≥1 year, were incubated and CYP3A content analyzed by immunoblotting exactly as detailed in (*a*). (*d*) Corresponding anti-Ub immunoblotting analyses of microsomes incubated as detailed in (c) were carried out exactly as detailed in Figure 3. (*e*) Liver microsomes (0 h) used in (*c*) and (*d*) above were further stored as suspensions at either –20°C or –80°C for 1 week before CYP3A immunoblotting analyses exactly as described in (*a*) above.

Figure 4*d*,*e* are observed if microsomes are stored for prolonged periods as pellets or even as suspensions for just a week. More importantly, comparative inspection of the immunoblots in Figures 3 and 4 clearly distinguishes the authentic CYP3A ubiquitination profiles (Figure 3*a* and *c*) from the CYP3A aggregation artifacts illustrated in Figure 4*d* by the absence of any immunochemically detectable CYP3A at the stacking/running gel interphase and/or bottoms of the gel wells (marked by an asterisk). A distinguishing feature of truly ubiquitinated CYP3A species is that they predominantly migrate to a region well above the 55 kDa parent protein but distinctly below the stacking/running gel interphase where CYP3A aggregates are usually found.

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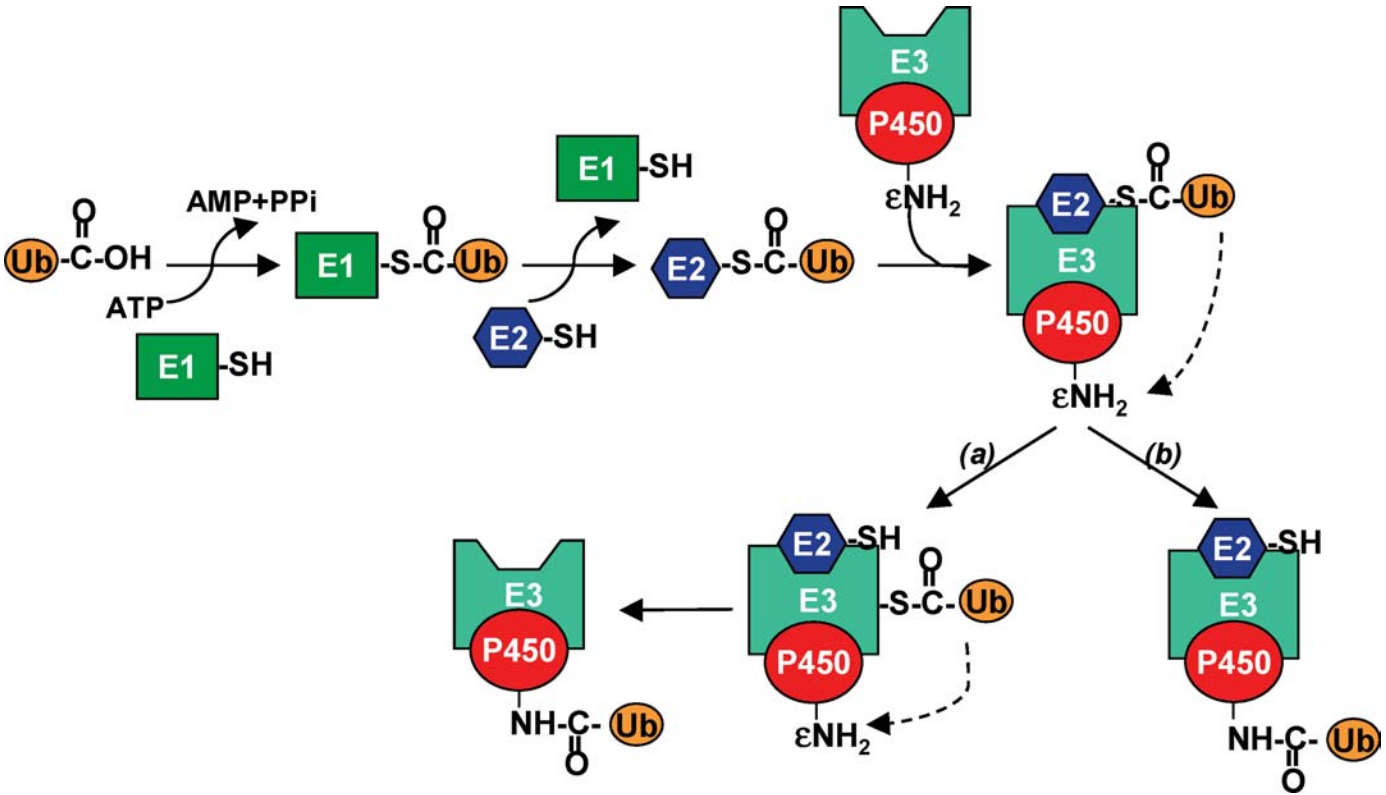

Figure 1 The cellular enzymatic machinery available for P450 ubiquitination. The roles of E1, E2, and E3 enzymes are discussed in the text. E2-substrate Ub-thioester relay via an E3-Cys residue (*a*) or directly (*b*) is shown.

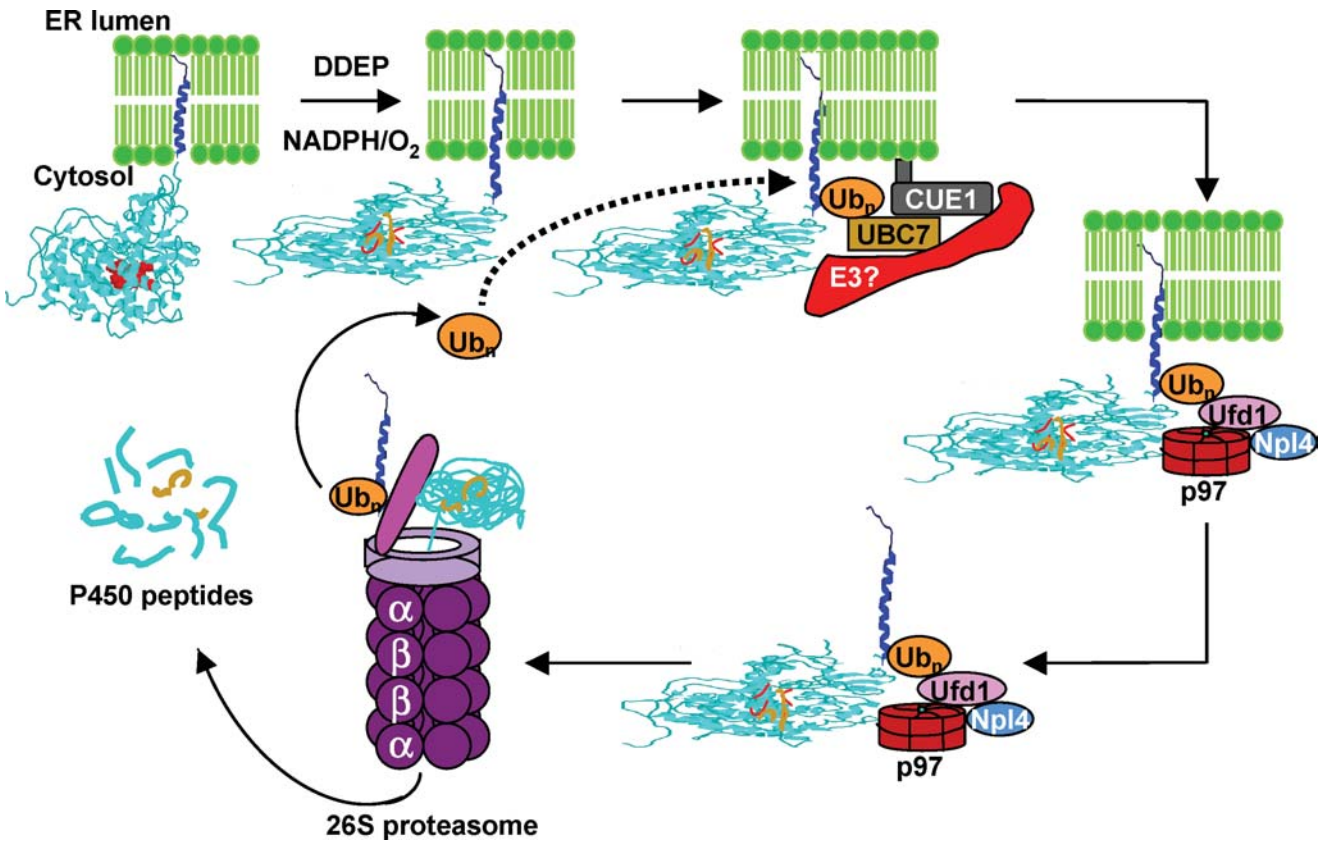

Figure 2 Hepatic P450 ERAD: Putative cellular participants. The plausible events in the Ub-dependent 26S proteasomal degradation of an ER-bound DDEP-inactivated CYP3A are illustrated. See the text for specific details.

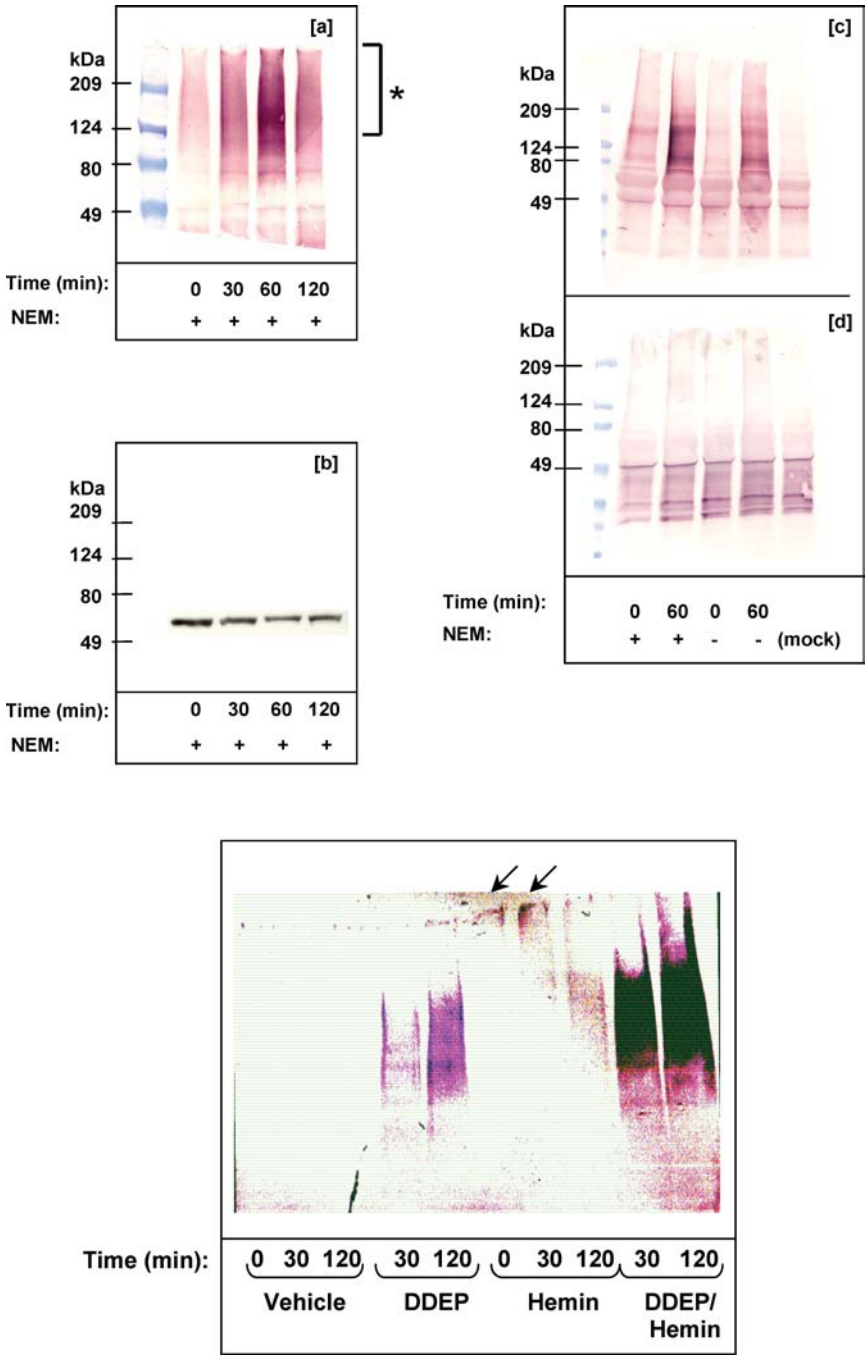

Figure 3 See legend on next page

Figure 3 Immunochemical detection of rat liver microsomal P450 ubiquitination after DDEP-treatment: effects of NEM, membrane autoclaving, and/or hemin. (*a*) Microsomes (500 μg) from DEX-pretreated rats given DDEP (125 mg/kg, ip) for 0, 30, 60, and 120 min were prepared from livers homogenized with (+) 5 mM NEM and stored at –80°C as pellets overlaid with 10% glycerol/0.1 M phosphate buffer, pH 7.4, until use within 2 weeks. Microsomes were resuspended and subjected to immunoprecipitation with goat polyclonal antirat CYP3A23 IgGs (3 mg) as described (12). The immunoprecipitated protein was solubilized by boiling in sample loading buffer consisting of final concentrations of 5% SDS, 25% glycerol, 50 mM DTT, and 2% β-mercaptoethanol in 150 mM Tris, pH 6.8 (100 μl). The supernatant (45 μl) containing the released P450 was subjected to anti-Ub immunoblotting analyses (ubiquitination) onto a 4–20% gradient Tris-HCl ready gel (BioRad). The electroblotted membranes were autoclaved at 120°C for 30 min, before blocking with 3% gelatin and overnight incubation with rabbit anti-Ub primary antibody (1:100, v/v; Sigma-Aldrich), followed by goat antirabbit alkaline phosphatase (AP)-conjugated secondary antibody (1:3000, v/v; BioRad). The membranes were extensively washed with hourly changes of Tris-buffered saline (TBS) for 5 h before color detection. (*b*) Liver microsomal aliquots (10 μg) obtained from the above treated rats were also subjected to immunoblotting analyses onto a 9% Tris-HCl gel. The extent of CYP3A degradation was assessed using a primary goat antirat CYP3A23 antibody (1:10,000, v/v) overnight, followed by a secondary rabbit antigoat horseradish peroxidase (HRP)-conjugated antibody (1:40,000, v/v) for 1 h followed by enhanced chemiluminescence (ECL) detection (113). (*c*) Livers of the rats given DDEP for 0 or 60 min [see (*a*)] were homogenized with (+) or without (–) 5 mM NEM. Microsomal aliquots (500 μg) were immunoprecipitated and the extent of ubiquitination analyzed exactly as described in (*a*). For reasons discussed, a "mock" immunoprecipitation without liver microsomes is included. (*d*) Identically electroblotted membranes were subjected to anti-Ub immunoblotting analyses without the autoclaving step. (*e*) Freshly isolated hepatocytes from untreated male rats were incubated with or without DDEP (0.5 mM), with or without hemin (100 μM) at 37°C for 0–120 min, exactly as described (12). Liver microsomes (1 mg) were immunoprecipitated with rabbit polyclonal antirat CYP2C11 antibodies. The CYP2C11 immunoprecipitates were subjected to anti-Ub immunoblotting analyses as in (*a*) above. Arrows indicate the absence of any P450 aggregates after hemin incubation of hepatocytes.

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