IDENTIFICATION OF A UBIQUITINATION-TARGET/SUBSTRATE-INTERACTION DOMAIN OF CYTOCHROME P-450 (CYP) 2E1

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ABSTRACT:
Cytochrome P-450 (CYP) 2E1, the alcohol-inducible form of CYP, metabolizes a wide variety of endogenous substrates, therapeutic agents, protocyticants, and procarcinogens. CYP2E1 levels are post-transcriptionally elevated in response to certain xenobiotic inducers (e.g., pyridine), and proposed mechanisms include increased translational efficiency and protection of the enzyme from ubiquitin-dependent proteolysis. Molecular modeling of a predicted cytosolic domain of CYP2E1 resulted in identification of a putative ubiquitination-target/substrate-interaction structure (residues 317–340). An affinity-purified antibody reactive to this domain quenched CYP2E1 ubiquitination in a concentration-dependent manner in a rabbit reticulocyte lysate-based ubiquitination assay. The same antibody also inhibited rat liver microsomal chlorzoxazone 6-hydroxylase activity, a marker of CYP2E1 catalytic activity, in an equivalent concentration-dependent manner. These two observations suggest an association between the CYP2E1 cytosolic domain involved in catalysis and its serving as a target for ubiquitination. Thus, these results provide a plausible mechanistic explanation for the observation that substrate binding shields the CYP2E1 protein from turnover by the ubiquitin-proteasome-dependent machinery.

Accelerated Communication

Cytochrome P-450 (CYP)1 2E1 metabolizes a wide variety of xenobiotics including low molecular weight therapeutic agents (e.g., isoniazid and acetaminophen), organic solvents (e.g., primary alcohols, acetone, carbon tetrachloride, and benzene), and procarcinogens (e.g., N-nitrosodimethylamine). Hepatic CYP2E1 expression is rapidly elevated in response to certain xenobiotics (e.g., ethanol and pyridine) and pathophysiological conditions (e.g., diabetes), and the combination of increased CYP2E1 levels and the presence of a protocytic or procarcinogen substrate predisposes a cell to injury (Lieber, 1997). CYP2E1 levels can be elevated by transcriptional or post-transcriptional mechanisms, the latter including increased translational efficiency (Kim and Novak, 1990; Kim et al., 1990), as well as mechanisms purported to produce protein stabilization (i.e., decreased turnover), in particular, inhibition of ubiquitin-mediated proteolysis (Tierney et al., 1992; Roberts et al., 1995; Korsmeyer et al., 1999).

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1 Abbreviations used are: CYP, cytochrome P-450; CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum.

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CYP2E1-catalyzed oxidation of carbon tetrachloride results in damage that facilitates rapid ubiquitination and degradation of the protein (Correia, 1991; Tierney et al., 1992). Roberts et al. (1995) and others have postulated that CYP2E1 is induced by substrate-mediated protection of the protein from ubiquitin-dependent proteolysis. Thus, in the presence of ethanol, CYP2E1 was shown to exhibit a biphasic half-life, suggesting a substrate-induced alteration in CYP2E1 conformation and resultant differential rates of turnover (Roberts et al., 1995). This observation, however, is challenged by other reports in which labeling of the protein and heme failed to yield biphasic degradation kinetics (Tsutsumi et al., 1993), and a more recent report by Roberts (1997) also failed in demonstrating CYP2E1 ubiquitination. Very recently, Korsmeyer et al. (1999) have clarified these issues by demonstrating that the CYPs do undergo ubiquitin-dependent 26S proteasomal degradation. However, little mechanistic information on ubiquitin-mediated CYP degradation is currently available.

Protein ubiquitination is a pathway by which many proteins are selectively degraded, and it plays a critical role in regulating cell division and differentiation, oncogenesis, apoptosis, DNA repair, signal transduction, membrane transport, and removal of abnormal proteins (Ciechanover, 1994; Ciechanover and Schwartz, 1998). Ubiquitination is a multicatalytic process that tags proteins with a chain of multiple ubiquitin moieties that are detected as high molecular mass smears that appear at the tops of SDS gels. This is followed by their selective degradation by the cytosolic 26S protease (the 26S proteasome). In ubiquitin-protein conjugates, the C-terminal carboxyl group of ubiquitin is joined to a single lysyl ε-amino group within an acceptor protein by an isopeptide bond. Additional ubiquitin groups
are added using ubiquitin-ubiquitin linkages to form a multubiquitin chain (Ciechanover and Schwartz, 1998).

In this study, we have sought to determine a ubiquitination-target/substrate-interaction domain of CYP2E1. Molecular modeling of CYP2E1 resulted in the identification of a putative ubiquitination-target/substrate-interaction structure that is located in the J-helix and J’ loop and is a structurally conserved region in the different CYP structures resolved to date (Graham and Peterson, 1999). Our experimental data implicate this domain to be on the cytoplasmic side of the microsomal membrane. An affinity-purified antidomain antibody reactive to this domain both quenched ubiquitination of CYP2E1 protein in the rabbit reticulocyte cell-free translation/ubiquitination system and inhibited rat liver microsomal CYP2E1 catalytic activity.

Materials and Methods

Generation of an Antipeptide Antibody Targeted toward the CYP2E1 Ubiquitination/Substrate-Interaction Domain. A 25-residue peptide (NCKYPEEEDHLHEIDRVSPGSRVPA-C; named 2E1- pep) was synthesized commercially by Research Genetics, Inc. (Huntsville, AL) and its purity was evaluated by mass spectrometry and HPLC (>90% pure). This synthetic peptide was cross-linked to Affigel-10 (Bio-Rad, Richmond, CA), a hydroxy-succinimide-activated Sepharose gel, at a concentration of 5 mg/ml gel. Affinity chromatography using this peptide-linked gel was used (Harlow and Lane, 1988) for the purification of anti-CYP2E1317–340 domain polyclonal antibody from an IgG pool that was prepared by immunizing goats with the whole rabbit CYP2E1 molecule (Kim et al., 1995).

Ubiquitination of CYP2E1 In Vitro. [35S]CYP2E1 was translated in vitro transcribed RNA using a plasmid containing a CYP2E1 insert encoding the full-length protein (provided by Dr. B.-J. Song, National Institute on Alcohol Abuse and Alcoholism; Song et al., 1986), using both the reticulocyte lysate and wheat germ lysate cell-free translation systems, according to the manufacturer’s protocols (Promega Inc., Madison, WI) in 50 µl volumes. Wild-type bovine ubiquitin (5 µM; Sigma Chemical Co., St. Louis, MO) was added to selected reactions, as was the proteasome inhibitor MG132 (Z-Leu-Leu-Leu-Val-chloromethyl ketone) (Harlow and Lane, 1988) for the purification of anti-CYP2E1317–340 domain polyclonal antibody from an IgG pool that was prepared by immunizing goats with the whole rabbit CYP2E1 molecule (Kim et al., 1995).

To test these hypotheses, we selected a 21-residue-long invariant sequence within this domain (Fig. 1A, shown in bold black font), and from our previous results on the high-scoring transmembrane segments, the predicted amino acid sequence (either anti-CYP2E1317–340 domain antibody or anti-total CYP2E1 antibody) could be aligned with a 12% SDS gel (Laemmli, 1970) and detected by fluorography. The intensity of bands representing unmodified and ubiquitinated CYP2E1 was measured by an ISi Gel Image Analysis instrument.

Measurement of Chlorozoxazone 6-Hydroxylase Activity. Chlorozoxazone 6-hydroxylase activity was measured in rat liver microsomes, essentially as described by Lucas et al. (1996). Reaction mixtures (total volume of 0.5 ml) containing 0.2 mg microsomes, 1 mM NADPH, and 10 to 50 µg antibody (either anti-CYP2E1317–340 domain antibody or anti-total CYP2E1 antibody), or an equivalent volume of antibody dilution buffer (50 mM sodium phosphate, pH 7.0), were incubated, with continual rotation, at room temperature for 30 min. The samples were then incubated at 37°C for 3 min before initiation of the reactions with 50 µM chlorozoxazine (estimated, in preliminary experiments, as the Kₐ in rat liver microsomes), and incubations were continued for 20 min. For comparison, additional samples were incubated with 50 µM diethyldithiocarbamate, a mechanism-based CYP2E1 inhibitor. For these incubations, microsomes were preincubated with diethyldithiocarbamate in the presence of NADPH for 15 min at 37°C before the addition of substrate. After incubation, samples were processed and analyzed for the formation of 6-hydroxycloazozone essentially as described (Lucas et al., 1996). Phenacetin (50 µM) was added to each sample as an internal standard. Final nitrogen-dried residues were dissolved in 50 µl of initial mobile phase, and 20 µl were separated by HPLC. Separations were achieved using a 3.9 × 150 mm C18 column (Waters Nova-pak) and a mobile phase gradient of 80% 0.5% glacial acetic acid in water/20% acetonitrile for 9 min and 25% 0.5% glacial acetic acid in water/75% acetonitrile for 9 min. Mobile phase flow rate was 1 ml/min, and ultraviolet absorption of the eluate was monitored at 287 nm. Under these conditions, 6-hydroxychlorozoxazine, phenacetin, and chlorozoxazone eluted with retention times of approximately 3.0, 7.3, and 11.8 min, respectively. The amounts of 6-hydroxychlorozoxazine that were formed were estimated by comparing 6-hydroxychlorozoxazine to phenacetin peak area ratios to those obtained in a standard curve, and enzyme activities were expressed as picomoles of 6-hydroxychlorozoxazine per minute per milligram protein. Each incubation was repeated three times, once in one set of rat liver microsomes, and twice in a second set of liver microsomes from a different rat. Statistical comparisons among treatment groups were performed using one-way ANOVA followed by Dunnett’s test. Control incubations performed in the absence of NADPH, substrate, or microsomes exhibited no detectable chlorozoxazone 6-hydroxylase activity.

Results

CYP2E1 has 37 Lysyl Residues: Prediction of a Substrate-Interaction/Ubiquitination Domain using Molecular Modeling. To narrow the range of the lysine residues for analysis of ubiquitination and formulation of a hypothesis, we generated molecular models of CYP2E1 using knowledge-based protein modeling methods (Peitsch, 1996). Initially, a theoretical model of the secondary structure of rat CYP2E1 (493 amino acids) was calculated using GCG software (Madison, WI) by the methods of Chou and Fasman (1978) and Kyte and Doolittle (1982). The high-scoring transmembrane segments were predicted by the SAPS program of Brendel et al. (1992) and the TMPred program of Hofmann and Stoffel (1993). From these computations, we developed a working model in which the CYP2E1 tertiary structure likely contains two major cytoplasmic domains, CD1 and CD2 (data not shown), which did not contain any predicted transmembrane sequences. Based on the three-dimensional structures of the soluble P-450 enzymes [Protein Data Bank entries 2HPD (Bacillus megaterium P-450 BM3 hemoprotein domain, Ravichandran et al., 1993), 1OXA (Succaropalyspora erythraea P-450 EryF, Cupp-Vickery and Polous, 1995), and 1FAG (P-450 BM3 heme domain complexed with the fatty acid substrate, palmitoleic acid, Li and Polous, 1997)], and on the theoretical model of the bovine cholesterol side chain cleavage P-450 (1SCC, Vijayakumar and Salerno, 1992), a molecular model for the CYP2E1 CD2 domain could be constructed using the Swiss-Model server (Peitsch, 1996; courtesy of Glaxo-Wellcome Trust, Geneva, Switzerland) running the ProMod software under the default set points. For comparative purposes, molecular models of the corresponding CD2 domains of CYP2B1, CYP1A1, CYP3A1, and CYP4A1 were also constructed. These models were refined more by energy minimization using CHARMM (Brooks et al., 1983), and they were then used to discern a variant loop structure within the various P-450 CD2 domains, which we hypothesized could account for differences in substrate specificity (data not shown). Figure 1A shows the amino acid residues that constitute the putative CYP2E1 CD2 domain (residues 286–460) that could be modeled (Fig.1B) because this is a structurally conserved region in the different CYPs (Graham and Peterson, 1999). The amino acid residues in the remainder of the CYP2E1 protein could not be modeled. The catalytic site Cys¹³⁷ that binds the heme-iron ligand is contained within this domain (Fig. 1A, shown in bold black font), and from our molecular model, is predicted to lie ~20 to 25 Å (distance between the α carbons) from the variant loop structure, which is within a reasonable range for bringing the heme-iron and a substrate together. Thus, we hypothesized that the variant structure contained within the CD2 domain may constitute a substrate interaction site. We further hypothesized that ubiquitination of CYP2E1 may occur on this same domain, which contains only two lysyl residues, Lys³¹⁷ and Lys³²⁴.

To test these hypotheses, we selected a 21-residue-long invariant peptide within this structure that is present in the human, rat, mouse, hamster, rabbit, pig, and bovine CYP2E1 (residues 317–338 of rat CYP2E1, demarcated by a black box in Fig. 1D) for development of
a specific antidomain antibody, to determine whether antibody binding to this sequence could inhibit CYP2E1 ubiquitination in vitro, and catalysis by the enzyme in rat liver microsomes.

**Generation of an Antipeptide Antibody Targeted toward the Putative CYP2E1 Ubiquitination/Substrate-Interaction Domain.** A 25 residue peptide (N-CKYPEIIEKLHEEIDRVIIGPSRVPAC; named 2E1-pep) was used for affinity purification of the anti-CYP2E1317–340 domain polyclonal antibody from a pool of anti-total CYP2E1 IgG. The cross-linking of 2E1-pep to BSA and to itself by glutaraldehyde is documented in Fig. 2A by using reducing SDS-polyacrylamide gel electrophoresis. These cross-linked 2E1-pep-derived products (CX-P) and rat liver microsomes were used in Western blotting experiments to verify the successful isolation of an anti-CYP2E1317–340 antibody (Fig. 2, B–D). The anti-total CYP2E1 antibody showed very faint immunoreactivity to CX-P (Fig. 2B; 2E1-pep-BSA cross-linked products are shown by the bracket), but strong reactivity to rat liver microsomal CYP2E1. By comparison, the affinity-purified anti-CYP2E1317–340 domain antibody exhibited a 1000-fold increased immunoreactivity to CX-P (Fig. 2C and D; 2E1-pep oligomers are shown by the solid upward arrows, and 2E1-pep-BSA cross-linked products are shown by the bracket), demonstrating the successful isolation an anti-CYP2E1317–340 antibody with very great avidity and specificity. This reagent was used to examine whether the protein domain reactive to this antibody was of significance for obtaining ubiquitination of the CYP2E1 protein in vitro and/or for allowing CYP2E1-mediated substrate catalysis in rat liver microsomes.

**Ubiquitination of CYP2E1 In Vitro.** [35S]CYP2E1 was translated from in vitro transcribed RNA, using both the reticulocyte lysate and wheat germ lysate cell-free translation systems. The reticulocyte lysate used in this assay contains ubiquitination enzymes and the 26S-proteasome, and has been shown to degrade multiubiquitinated proteins (Haas and Bright, 1988). Figure 3 demonstrates that ubiquitination of the newly translated and free CYP2E1 proceeds at a rapid rate in reticulocyte lysate. The amount of polyubiquitinated CYP2E1 peaked at about 30 min (Fig. 3, lane 2, bracketed) and subsequently diminished at 60, 120, and 180 min (Fig. 3, lanes 2–5 and 6–8). It has been shown that only the polyubiquitin chains containing more than three to four ubiquitin moities (indicated by arrows in Fig. 3) are of significance in targeting the tagged protein for proteasomal degradation (Ciechanover, 1994). Inhibition of the 26S proteasome activity by MG132 (Fig. 3, lanes 2–5 and 6–8). 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ubiquitinated forms of CYP2E1 were formed in a time-dependent manner and were targeted for degradation by the 26S proteasome when CYP2E1 mRNA was translated in vitro using the reticulocyte lysate cell-free system. By contrast, CYP2E1 did not become multi-ubiquitinated when CYP2E1 mRNA was translated using the wheat germ lysate system, which lacks the ubiquitin-conjugating machinery (Fig. 3, lanes 11–14) relevant for CYP2E1 ubiquitination.

Inhibition of CYP2E1 Ubiquitination In Vitro by the CYP2E1 317–340 Domain-Specific Antibody. Having established the time course of maximal polyubiquitination of \( ^{[35}S \)CYP2E1 in reticulocyte lysate (i.e., 30 min), we next examined whether incubation with the anti-CYP2E1 317–340 domain antibody would inhibit this process (Fig. 4). From our model, we hypothesized that cotranslational binding of the anti-CYP2E1 317–340 antibody to the CYP2E1 317–340 domain structure, containing the putative lysyl residues targeted for ubiquitination, would prevent the ubiquitination of free CYP2E1. The addition of increasing amounts (0.02–2 \( \mu \)g) of anti-CYP2E1 317–340 domain antibody to in vitro translation reactions (Fig. 4, lanes 2–6) produced a concentration-dependent decrease in the amount of polyubiquitinated CYP2E1 (indicated in the Fig. 4 fluorogram by brackets at the top of the lanes) relative to that detected in an incubation containing 2 \( \mu \)g of anti-total CYP2E1 antibody (Fig. 4, lane 1). The anti-CYP2E1 317–340 antibody-mediated decrease in the amount of polyubiquitinated CYP2E1 is a very significant decrease for this event, based on other studies (Levitskaya et al., 1997). Thus, these results support a critical role for the CYP2E1 317–340 domain structure in CYP2E1 ubiquitination.

Inhibition of CYP2E1 Catalytic Activity by the CYP2E1 317–340 Domain-Specific Antibody. Finally, from our model, we hypothesized that the CYP2E1 domain that was targeted for ubiquitination would be the same as that involved in substrate interaction. Thus, we predicted that the same anti-CYP2E1 317–340 antibody that inhibited CYP2E1 ubiquitination would also inhibit CYP2E1 catalytic activity. To examine this possibility, rat liver microsomes were incubated with the anti-CYP2E1 317–340 antibody, or with anti-total CYP2E1 IgG, and chlorzoxazone 6-hydroxylase activity was measured as a marker of CYP2E1 enzymatic activity. Incubation of rat liver microsomes with 50 \( \mu \)g of the anti-total CYP2E1 IgG had no significant effect on chlorzoxazone 6-hydroxylase activity. By comparison, incubation of microsomes with 10 or 50 \( \mu \)g of the anti-CYP2E1 317–340 antibody produced a significant \( (P < .01) \) and concentration-dependent inhibition in chlorzoxazone 6-hydroxylase activity (Fig. 5A). The concentrations of the anti-CYP2E1 317–340 antibody that inhibited microsomal enzymatic activity (i.e., 0.02 or 0.1 \( \mu \)g/\( \mu \)l) were in the same range as those that inhibited ubiquitination during in vitro translation in reticulocyte lysates. The maximal inhibition observed after incubation with the anti-CYP2E1 317–340 antibody (55%) was at least as great as the percentage of inhibition (47%) that was produced after incubating rat liver microsomes with 50 \( \mu \)M diethylthiocarbamate, a mechanism-based CYP2E1 inhibitor (Fig. 5B), suggesting that incubation with the anti-CYP2E1 317–340 antibody produced a very effective inhibition of CYP2E1 catalytic activity.

Discussion

The role of ubiquitination in substrate-inducible CYP2E1 expression is controversial. Thus, Roberts et al. (1995) and others (Eliasson et al., 1988) have postulated that CYP2E1 is induced by substrate-mediated stabilization of the protein from proteolysis. By this mechanism, the presence of substrate would cause an allosteric change that would render the enzyme less susceptible to ubiquitin-dependent turnover by shielding the domain that is the target of ubiquitination. Although reasonable, this proposition has been challenged by other findings (Tsutsumi et al., 1993), including a recent report by Roberts (1997). In the present study, we have sought to determine a biochem-

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A, lane 1 shows molecular mass markers; lane 2 contains the synthetic CYP2E1 317–340 peptide (2E1-pep, 2 \( \mu \)g); lane 3, BSA, 5 \( \mu \)g; lane 4, 2E1-pep and BSA before cross-linking (total 3 \( \mu \)g); lanes 5 and 6, the products after cross-linking of 2E1-pep and BSA (lane 6 has 3 times (9 \( \mu \)g) the total amount of protein as lane 5, 3 \( \mu \)g). The 15% SDS gel was stained with Coomassie Brilliant Blue R250. B, Western blot using anti-total CYP2E1 IgG (60 \( \mu \)g IgG/30 ml; 2 h. incubation in primary antibody); lane CX-P, cross-linked 2E1-pep-BSA products as seen in (A), lane 5; lane M, 20 \( \mu \)g of rat liver microsomal protein. C, same as (B), but the blot was processed with the anti-CYP2E1 317–340 domain antibody (60 \( \mu \)g IgG/30 ml; 2 h. incubation in primary antibody). D, same as (C) but the blot was exposed for a longer time (20X) than that in (C) to show the full extent of the anti-CYP2E1 317–340 domain antibody reaction, particularly immunoreactivity to the 2E1-pep oligomers (shown by the solid upward arrows; please compare B, C, and D). In (D), lane M, the slower migrating immunoreactive material is probably ubiquitinated CYP2E1 in microsomes. Immunoreactive bands were visualized by enhanced chemiluminesence and exposure to X-ray film.

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Fig. 2. Generation of antidomain antibody to the putative ubiquitination/substrate-interaction domain of CYP2E1.
In vitro Ubiquitination Assay of CYP2E1

Lanes 1 through 10 show fluorograms of [35S]CYP2E1 after in vitro translation of CYP2E1 mRNA in rabbit reticulocyte lysate for various periods of time. Lanes 2 through 5 show in vitro translated CYP2E1 in various states of ubiquitination. Note that at times of 120 and 180 min (lanes 4 and 5), there is almost no unubiquitinated CYP2E1 remaining in the reaction because polyubiquitinated protein, as shown by the {brackets}, is rapidly degraded with time. Reactions shown in lanes 6 through 14 were supplemented with 5 μM wild-type ubiquitin (Ub; to ensure that ubiquitin was not a rate-limiting factor; Banerjee et al., 1993). Lanes 9 and 10 show stabilized CYP2E1-Ub conjugates, when the proteasome inhibitor MG132 (20 μM) was added to the translation reactions. Lanes 11 through 14 show the results that were obtained after CYP2E1 mRNA was translated in wheat germ lysate, with no visible ubiquitination at 120 min. MG132 addition to these reactions had no effect (lanes 13 and 14).

Inhibition of CYP2E1 Ubiquitination in vitro

Fluorogram lanes 1 through 6 show in vitro translation/ubiquitination reactions with CYP2E1 as described in Fig. 3, with all reactions conducted for 30 min. The lane 1 reaction contained 2 μg of anti-total CYP2E1 antibody, and served as a control for the reactions shown in the remaining five lanes, which contained amounts of anti-CYP2E1 317–340 antibody ranging from 0.02 μg (lane 2) to 2 μg (lane 6). The results show a progressive decrease in the amount of polyubiquitinated CYP2E1 (indicated by brackets) as increasing amounts of the anti-CYP2E1 317–340 antibody is added to the reactions. Note that at 1 and 2 μg (lanes 5 and 6, respectively), the anti-CYP2E1 317–340 antibody is capable of decreasing the level of ubiquitinated CYP2E1 that is formed (arrows).

A specific basis for the ability of a CYP2E1 substrate to protect the protein from ubiquitin-mediated proteolysis. Because the CYP2E1 protein contains 37 lysyl residues, and each of the cytoplasmically exposed lysyl residues could be considered a candidate for ubiquitin conjugation, we wished to apply some rational basis for targeting a small subset of these residues for study. Because no structural information is currently available on CYP2E1, molecular modeling was performed. Although the precise structures required for detailed studies
The effects of incubating rat liver microsomes with 0.02 or 0.1 μg/μl anti-CYP2E1P317–340 antibody (Anti-domain) or with 0.1 μg/μl anti-total CYP2E1 antibody (anti-2E1) on chlorzoxazone 6-hydroxylase activity were determined, as described in Materials and Methods (A). For comparison, the effect of incubating rat liver microsomes with 50 μM diethylthiocarbamate (DDC) on chlorzoxazone 6-hydroxylase activity was also determined (B). For each panel, the left axis presents the enzymatic activities as picomoles 6-hydroxylchlorzoxazone per minute per milligram microsomal protein, whereas the right axis presents the data as percentages of control activities. Each bar represents the mean ± S.D. chlorzoxazone 6-hydroxylase activity determined in three independent microsomal incubations, using microsomes prepared from the livers of two different rats. *Significantly different from control, P < .01.

of protein-ligand interaction can only be obtained experimentally, theoretical protein modeling, such as we have used, provided us with “low-resolution” models of the spatial arrangement of important amino acid residues and protein domains. We emphasize that the models served solely as the rational basis for generation of a hypothesis that could be tested empirically. In this case, molecular modeling permitted the identification of a putative ubiquitination domain at residues 317–340, which we hypothesized may serve as both a substrate interaction domain and a target site for ubiquitination. To test this hypothesis, we prepared an antibody directed against this domain, and determined the ability of the antibody to interfere with CYP2E1 ubiquitination in vitro and with CYP2E1 catalytic activity in rat liver microsomes. Our results, which showed inhibition of both phenomena, supported our hypothesis and the general accuracy of the CYP2E1 molecular model. In particular, the catalytic data support the conclusion that the 317–340 domain is cytosolic, and is not buried within the endoplasmic reticulum membrane. In connection with the microsomal CYP2E1 experiments, it is important to point out that the anti-CYP2E1P317–340 antibody used in our study did not inhibit catalysis by affecting the P-450 reductase binding site on CYP2E1 as that site is situated within the endoplasmic reticulum membrane. In connection with the microsomal CYP2E1 experiments, it is important to point out that the anti-CYP2E1P317–340 antibody used in our study did not inhibit catalysis by affecting the P-450 reductase binding site on CYP2E1 as that site is situated between SRS-4 and SRS-5 in that scheme. Molecular dynamics simulations with P-450cam and P-450 BM3 have shown that substrate binding and metabolite release actuate motions in the catalytic domain of these proteins (reviewed in Graham and Peterson, 1999). It has been proposed that the control of protein dynamics to allow metabolism of specific substrates at the catalytic site is coordinated in individual CYPs according to the particular substrates on which it acts, the efficiency of the reaction, and the region- and stereo-selectivity required (Wade et al., 1998). This necessitates that the substrate interaction domains in the CYPs be physically more expansive than the actual substrate-tethering site.

Ubiquitination plays a role in the breakdown of improperly folded and damaged proteins (Ciechanover, 1994; Ciechanover and Schwartz, 1998). All newly synthesized proteins are not in their final conformation. One may argue that these proteins are somehow functionally and spatially protected from degradation. The other possibility is that the cell makes them in large excess to permit a sufficient fraction of them to reach their final destination in the membrane. It has been shown that the majority (~75% of the total protein synthesized) of cystic fibrosis transmembrane conductance regulator (CFTR) precursors fail to insert in the endoplasmic reticulum (ER) and are rapidly degraded by ubiquitin-dependent proteolysis (Ward et al., 1995). Moreover, a mutant CFTR causing cystic fibrosis, AF508, which cannot fold properly, is degraded in almost its entirety (>99%) by this same pathway (Ward et al., 1995). It is to be noted that these CFTR proteins that are degraded do not reach their final destination in the membrane and the conformation of these proteins that is recognized by the ubiquitination machinery is distinct from those that are inserted in the membrane. Recently, Sato et al. (1998) have demonstrated that for the CFTR protein cotranslational protein ubiquitination can occur while it is still attached to the ribosome. The implications of such modification for the CFTR protein is reviewed in Kopito (1999). Based on these observations and our results presented here, it is postulated that the newly synthesized uninserted form of CYP2E1 would be targeted by a similar ubiquitin conjugation mechanism.

Turnover of the microsomal form of CYP2E1 would likely demand a different variety of substrate recognition machinery and also a different type of ubiquitin-ligation complex than would degrade the free CYP2E1. An endoplasmic reticulum-bound ubiquitin-conjugating enzyme (E2) has been described in yeast (Sommer and Jentsch, 1993). This E2, Ubc6, has been postulated to function in the degradation of endoplasmic reticulum-associated proteins, and a homolog of this enzyme would be a candidate for the processing of microsomal CYP2E1. Recently, it has been demonstrated in yeast that degradation of endoplasmic reticulum-bound proteins occurs by the ubiquitin-proteasome system via retrograde transport of the substrate protein after its ubiquitination on the cytosolic surface (Miller et al., 1996). Additional work has shown the targeting of another soluble yeast E2, Ubc7, to the endoplasmic reticulum, caused by its assembly with the Cuel protein. These findings define the endoplasmic reticulum cytosolic surface as a cellular “ubiquitin conjugation platform” (Biederer et al., 1997), whereby assembly of the ubiquitin-proteasome pathway components could increase the efficiency of ubiquitin-dependent proteolysis.

A good example for this kind of ubiquitination pathway is the ER membrane protein HMG-Co A reductase, the rate-limiting enzyme for cholesterol synthesis, that has been shown to be degraded by ubiquitination under conditions of high cholesterol levels (Hampton et al., 1996). Once tagged, degradation can follow as proteasomes have been shown to be associated with the ER membrane (Goldberg and Rock, 1992). Other cases of degradation of membrane proteins by the ubiquitin-proteasome pathway have been reported recently. Inhibitors of the 26S proteasome have been shown to prevent the cotranslational...
degradation of Apolipoprotein B100 (apoB100) in HepG2 cells (Be- 
oisn and Grand-Perret, 1997). Targeting of this secretory protein to
the 26S proteasome is regulated by the chaperone Hsp70 and the
binding of apoB100’s lipid ligands (Fisher et al., 1997).

To proceed toward understanding the post-translational regulation
of CYP2E1, there is a need for a comprehensive study of the turnover
and trafficking of free as well as microsome-associated forms.

Recently, Anandatheerthavarada et al. (1999) and Bhagwat et al. (1999)
have demonstrated that CYPs are indeed trafficked to the microsomes
or to the mitochondria in a regulated manner. In our initial studies,
ubiquitination of CYP2E1 synthesized in vitro was examined, rather
than ubiquitination of microsome-bound CYP2E1. This study brings
out the point that there are likely two subtypes of ubiquitination
systems involved in the turnover of CYP2E1, one acting on newly-
synthesized forms and the other on microsomal forms. Information
obtained from these studies will be invaluable in understanding the
mechanism(s) regulating P-450 turnover and how exposure to
xenobiotics affect this process.

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