Regulation of Cytochrome P450 2E1 by Heat Shock Protein 90-Dependent Stabilization and CHIP-Dependent Proteasomal Degradation†

Yoshihiro Morishima, Hwei-Ming Peng, Hsia-lien Lin, Paul F. Hollenberg, Roger K. Sunahara, Yoichi Osawa, and William B. Pratt*

Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan 48109

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ABSTRACT: Alcohol-inducible cytochrome P450 2E1 (CYP2E1) has the most rapid turnover of any member of this large family of membrane-bound oxygenases, and its degradation rate is altered profoundly by various substrates, such as ethanol and CCl_4. CYP2E1 is degraded by the ubiquitin−proteasome pathway, and because the hsp90/hsp70-based chaperone machinery is often involved in maintaining the balance between protein integrity and degradation by this pathway, we have asked whether CYP2E1 is regulated by the chaperone machinery. We show here that treatment of transformed human skin fibroblasts stably expressing CYP2E1 with the hsp90 inhibitor radicicol results in CYP2E1 degradation that is inhibited by the proteasome inhibitor lactacystin. Immunoadsorption of hsp90 from cytosol of HEK cells expressing the truncated CYP2E1(Δ3−29) yields coadsorption of CYP2E1(Δ3−29). Cotransfection of HEK cells with both the truncated CYP2E1 and the hsp70-dependent E3 ubiquitin ligase CHIP results in CYP2E1-(Δ3−29) degradation, and CYP2E1(Δ3−29) co-immunoadsorbs with myc-CHIP from cytosol of cotransfected cells. Purified, bacterially expressed CYP2E1(Δ3−29) is ubiquitylated in a CHIP-dependent manner when it is incubated with a purified system containing the E1 ubiquitin activating enzyme, E2, and CHIP. CYP2E1 is the first P450 shown to be an hsp90 "client" protein that can be ubiquitylated by the hsp70-dependent E3 ubiquitin ligase CHIP. Our observations lead to a general model of how substrates, such as ethanol, can regulate the interaction of CYP2E1 with the chaperones hsp90 and hsp70 to profoundly alter enzyme turnover.

The cytochrome P450s constitute a large family of heme proteins that catalyze the oxidation of endogenous substrates, such as steroids, and exogenous compounds, such as drugs, toxicants, and procarcinogens. The ethanol-inducible CYP2E1 is the most rapidly degraded of the P450s, having a rapid phase half-life of 6−7 h in the absence of substrate (*1*). In the presence of certain substrates, such as ethanol, the enzyme is stabilized, and stabilization is a major mechanism by which CYP2E1 is induced (*1*, *2*). In the presence of suicide substrates, such as CCl_4, CYP2E1 is rapidly degraded (*3*, *4*). Rapid phase, mechanism-based inactivation of CYP2E1 is NADPH-dependent, and its subsequent degradation occurs via the proteasomal pathway (*2*−*7*). Several studies have shown that loss of CYP2E1 is associated with ubiquitylation of the enzyme (*2*, *4*, *8*), although in some reports ubiquitylation was not observed (*3*, *6*). A central problem in the field of protein turnover is how proteins that are damaged are sorted into the ubiquitin−proteasome pathway of degradation. There is substantial evidence with a variety of signaling proteins that chaperones hsp90[1] and hsp70 play a key role in the balance between the maintenance of protein integrity and degradation by the ubiquitin−proteasome pathway (*9*, *10*). Inasmuch as CYP2E1 is degraded by this pathway in a manner that can be manipulated by substrates, it may prove to be a useful system for studying how such triage decisions are made.

Hundreds, perhaps thousands, of cellular proteins are chaperoned by the hsp90/hsp70-based chaperone machinery (*11*), and we have previously shown that hsp90 and hsp70 are associated with neuronal nitric oxide synthase (nNOS), a cytosolic, P450-like, heme protein (*12*). Dynamic assembly of client protein·hsp90 complexes by the chaperone machinery stabilizes client proteins, and inhibition of hsp90 binding by geldanamycin or radicicol uniformly results in client protein destabilization manifest as degradation via the ubiquitin−proteasome pathway (*10*). Inhibition of hsp90 function by geldanamycin or radicicol and suicide inactivation of nNOS by certain substrates both lead to proteasomal degradation of the enzyme (*12*−*14*). nNOS is ubiquitylated prior to its degradation by the proteasome (*13*, *14*), and we have found that the chaperone-dependent E3 ligase CHIP can act as a ubiquitin ligase for nNOS to initiate its degradation (*15*). CHIP is a U-box-containing E3 ubiquitin ligase that binds through its tetratricopeptide repeat (TPR)

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* To whom correspondence should be addressed: Department of Pharmacology, The University of Michigan Medical School, 1301 Medical Science Research Building III, Ann Arbor, MI 48109-0632. Telephone: (734)764-5414. Fax: (734)763-4450. E-mail: ymo@umich.edu.

[1] Abbreviations: hsp, heat shock protein; CHIP, carboxyl terminus of hsp70-interacting protein; Ub, ubiquitin; GST−Ub, glutathione *S*-transferase-tagged ubiquitin; TPR, tetratricopeptide repeat; nNOS, neuronal nitric oxide synthase; TPMT, thiopurine *S*-methyltransferase; Hop, Hsp-organizing protein.

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domain to TPR acceptor sites on hsp90 and hsp70 (*16*, *17*). CHIP has been shown to facilitate the ubiquitylation of hsp90 client proteins, such as the glucocorticoid receptor (*18*), the cystic fibrosis transmembrane conductance regulator protein (*19*), and Raf-1 kinase (*20*).

The notion with respect to nNOS degradation is that suicide inactivation of the enzyme alters the substrate−heme binding cleft such that hydrophobic regions that normally lie in the cleft interior are exposed on the surface of the enzyme. The initial triage step ocuurs via binding of hsp70 to the exposed hydrophobic region, with CHIP then binding to the nNOS-bound hsp70 to initiate ubiquitylation followed by proteasomal degradation (*15*). In the absence of substrate-induced damage, hsp70 binds to the hsp90 client proteins to assemble a client protein·hsp70 complex that is primed to accept the Hop (Hsp-organizing protein) component of the hsp90/hsp70-based chaperone machinery (*11*). Hop binds via an N-terminal TPR domain to hsp70 and via a central TPR domain to hsp90 (*21*) to bring the two chaperones together in a machinery that forms the client protein·hsp90 complex (*11*), stabilizing it from degradation. Hop and CHIP compete for binding to the TPR acceptor site on hsp70 (*22*), and there is some, as yet undefined, difference in the conformation of the hsp70 bound to suicide-inactivated nNOS that favors CHIP binding and ubiquitylation versus assembly of a complex with hsp90 and stabilization (*15*).

In this study, we report that treatment of cells stably expressing CYP2E1 with the hsp90 inhibitor radicicol leads to CYP2E1 degradation that is inhibited by the proteasome inhibitor lactacystin. Transient transfection of CHIP into HEK cells causes loss of CYP2E1, and CYP2E1(Δ3−29) co-immunoadsorbs with myc-CHIP from the cytosol of cotransfected cells, suggesting that CHIP binds to P450·chaperone complexes in vivo. In a purified system containing the E1 ubiquitin activating enzyme, E2, and CHIP, both purified CYP2E1(Δ3−29) and CYP2B4(Δ2−27) are ubiquitylated, and the ubiquitylation of CYP2E1(Δ3−29) is shown to be CHIP-dependent. These observations show that CYP2E1 is stabilized by hsp90 and that it is subject to CHIP-dependent ubiquitylation. This is the first P450 shown to be stabilized by hsp90 and to undergo CHIP-dependent ubiquitylation.

MATERIALS AND METHODS

Materials. SV40 transformed human skin fibroblasts stably transfected with the full-length rat CYP2E1 (GM2E1 cells) were described previously (*23*). Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen Corp. (Grand Island, NY), and α-MEM was from BioWhittaker. 7-Ethoxycoumarin and 7-hydroxycoumarin were purchased from Aldrich Chemical Co. (Milwaukee, WI). Radicicol and dilauroyl-L-α-phosphatidylcholine (C12:O) were purchased from Sigma. Goat anti-human CYP2E1 serum was from Oxford Biochemical Research (Oxford, MI). Sheep anti-rabbit CYP2E1 and goat anti-rabbit CYP2B4 antibodies were provided by J. Coon (The University of Michigan Medical School). The affinity-purified IgG used for immunoblotting ubiquitin was from Dako Corp. (Carpinteria, CA). Rabbit anti-CHIP antibody was from Affinity BioReagents (Golden, CO). The N27F3-4 anti-72/73-kDa hsp monoclonal IgG (anti-hsp70) used for immunoblotting hsp70 was from StressGen Biotechnologies (Victoria, BC); the BB70 monoclonal IgG used for immunoadsorbing hsp70 was provided by D. Toft (Mayo Medical School, Rochester, MN), and the 8D3 monoclonal IgM used to immunoadsorb hsp90 was provided by G. Perdew (The Penn State University, State College, PA). Lactacystin was purchased from BIOMOL (Plymouth Meeting, PA). Glutathione *S*-transferase (GST)-tagged ubiquitin, ubiquitin activating enzyme (E1), and GST-tagged UbcH5a were from Boston Biochem (Cambridge, MA). Plasmids expressing amino-terminal truncations of CYP2E1(Δ3−29) and CYP2B4(Δ2−27) were described by Pernecky et al. (*24*) and obtained from the laboratory of J. Coon, as was purified NADPH-cytochrome P450 reductase. The cDNA for expressing the UbcH5a−GST fusion protein was kindly provided by C. M. Pickart (Johns Hopkins Medical School, Baltimore, MD). pcDNA3 plasmids for expressing CHIP and myc-CHIP (*17*, *25*) were kindly provided by C. Patterson (University of North Carolina, Chapel Hill, NC). The pCMV2E1 plasmid for expressing full-length rat CYP2E1 has been described previously (*23*).

Cell Culture. GM2E1 cells were grown as monolayer cultures in α-MEM containing 10% calf serum in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. Human embryonic kidney (HEK) 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum. For transfection, 293T cells in 75 cm^2 flasks at ∼60% of confluence were incubated for 3 h with 10 mL of serum-free DMEM, and the medium was aspirated. They were then incubated with 2 mL of transfection mix [3 μL of TransFast transfection reagent (Promega) per microgram of DNA in 2 mL of DMEM]; after 1 h, 10 mL of DMEM with 10% fetal bovine serum was added, and the incubations were continued for 48 h. Cells were transfected with 10 μg of CYP2E1(Δ3−29), 3 μg of CYP2E1, 5 μg of CHIP, or 5 μg of myc-CHIP cDNA per flask.

Cytosol Preparation and Immunoadsorption. After being transfected for 48 h, cells were harvested and washed in Hank's buffered saline solution. The cells were resuspended in 1.5 volumes of HEM buffer [10 mM HEPES (pH 7.5), 1 mM EDTA, and 20 mM sodium molybdate] containing 1 tablet per 10 mL of Complete Mini protease inhibitor and 1 mM phenylmethanesulfonyl fluoride, and disrupted by five cycles of freezing and thawing in dry ice to prepare the total cell lysate. In some experiments, this lysate was further centrifuged at 100000*g* for 15 min, and the resultant supernatant, termed cytosol, was collected, flash-frozen, and stored at −80 °C. For immunoadsorption of hsp90, 200 μL aliquots of cytosol from HEK293T cells transfected with CYP2E1(Δ3−29) cDNA were incubated at 4 °C for 2 h with 25 μL of IgG against IgM and 25 μL of nonimmune IgM or 8D3 monoclonal anti-hsp90 IgM in the presence of 14 μL of protein A-Sepharose. For immunoadsorption of myc-CHIP, 200 μL aliquots of cytosol from HEK293T cells cotransfected with CYP2E1(Δ3−29) cDNA and myc-CHIP cDNA were incubated at 4 °C for 2 h with 6 μL of nonimmune IgG (6 μg) or 30 μL of 9E10 anti-myc antibody (Santa Cruz sc-40, 6 μg) and 14 μL of protein A-Sepharose. The immune pellets were washed four times with 1 mL of TEGM buffer [10 mM TES (pH 7.6), 50 mM NaCl, 4 mM EDTA, 10% (w/v) glycerol, and 20 mM sodium molybdate], and immunoadsorbed proteins were resolved by electrophoresis and Western blotting.

Assay for 7-Ethoxycoumarin O-Deethylase Activity. To assay CYP2E1 activity in intact cells, GM2E1 cells were seeded in 60 mm dishes ($1-2 \times 10^6$ cells) 1 day prior to the assay (*26*). The next day, radicicol, lactacystin, or DMSO vehicle was added to 2 mL of fresh medium, and after the indicated times of incubation, the medium was removed and replaced with 1 mL of DMEM with 100 μM 7-ethoxycoumarin. After incubation for 2 h, the medium was removed and mixed with one-fifth volume of 2.3 M glycine-NaOH buffer (pH 10.3), and the 7-ethoxycoumarin *O*-deethylase activity was evaluated by measuring the fluorescence (390 nm excitation and 440 nm emission) of 7-hydroxycoumarin on a Shimadzu RF-5301PC spectrofluorometer. A standard curve was generated by adding known amounts of 7-hydroxycoumarin to the assay medium and assaying as described above. To determine the total amount of cell protein, cells were removed from the dishes with trypsin, rinsed with 1 mL of DMEM, suspended in a hypotonic solution (0.2 mM $MgSO_4$, 0.38 mM KH_2PO_4, 0.61 mM Na_2HPO_4, and 1 mM DTT) and sonicated. Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard. CYP2E1 activity is expressed as picomoles of 7-hydroxycoumarin produced per minute per milligram of cell protein.

In Vitro Ubiquitylation of CYP2E1 and 2B4. To conjugate Ub to CYP2E1(Δ3−29) or CYP2B4(Δ2−27), 0.25 μg of purified P450 was incubated for 2 h at 30 °C with a purified system containing E1 ubiquitin activating enzyme (0.1 μM), E2 UbcH5a (1.5 μM), His-tagged CHIP (4.0 μM), GST-tagged ubiquitin (8.3 μM), 1 mM DTT, and an ATP-generating system consisting of 4 mM ATP, 20 mM creatine phosphate, 10 mM $MgCl_2$, and 20 units/mL creatine phosphokinase, expressed as final concentrations, adjusted to a total volume of 20 μL with 50 mM Tris-HCl (pH 7.5). After incubation, 20 μL of sample buffer was added, and a 15 μL aliquot was analyzed by Western blotting with sheep anti-rabbit CYP2E1, anti-Ub, and goat anti-rabbit CYP2B4.

In some studies, CYP2E1(Δ3−29) was pretreated with purified hsp70 and hsp40 and then ubiquitylated as described above. In these experiments, 5.0 μM hsp70 and 0.5 μM hsp40 were incubated at 30 °C for 5 min with 5 μM CYP2E1 and the ATP-generating system adjusted to a total volume of 25 μL with HKD buffer [10 mM Hepes (pH 7.4), 100 mM KCl, and 5 mM DTT]. After sitting on ice for 15 min, the reaction mixture was diluted 5-fold with HKD buffer. An aliquot (5 μL) of this reaction mixture was substituted for P450 in the ubiquitylation reaction mixture containing the purified ubiquitin ligases described above. The proteins were resolved by SDS−polyacrylamide gel electrophoresis and immunoblotted for CYP2E1.

In some studies, CYP2E1(Δ3−29) was incubated under catalytic conditions with CCl_4 prior to the ubiquitylation reaction. In these experiments, 0.5 μM CYP2E1(Δ3−29) was mixed for 3 min at room temperature with 30 μM L-α-phosphatidylcholine and 1 μM purified NADPH-cytochrome P450 reductase. The mixture was placed on ice, and 5.0 μM hsp70, 0.5 μM hsp40, the ATP-generating system, and HKD buffer were added. To this were added 2 mM CCl_4 and an NADPH-generating system to achieve a 50 μL mixture that was incubated for 20 min at 30 °C. Ten microliters of this incubation was added to 40 μL of the in vitro ubiquitylation mixture for a further incubation of 1 h at 30 °C.

Expression and Purification of P450s, CHIP, hsp70, hsp40, and UbcH5a. Rabbit CYP2E1(Δ3−29) and CYP2B4(Δ2−27) were expressed in *Escherichia coli* and purified as described previously (*27*, *28*). His-CHIP was bacterially expressed and purified by Ni−NTA affinity chromatography as previously described (*16*). Hsp70 was purified from rabbit reticulocyte lysate by sequential chromatography on DE52, hydroxylapatite, and ATP−agarose columns as described previously (*29*). YDJ-1, the yeast ortholog of hsp40, was expressed in bacteria and purified by sequential chromatography on DE52 and hydroxylapatite as described previously (*30*). The UbcH5a−GST fusion protein was expressed and purified without the GST tag as described previously (*31*).

Plasmid Construction. A pcDNA3.1 expression construct encoding the truncated CYP2E1(Δ3−29) was generated from rabbit cytochrome P450 clone pJL3aS (*32*). pJL3aS was digested with Xba and HindIII. The resulting 1.5 kb DNA fragment was gel isolated, and then inserted into the pcDNA3.1(+) vector previously digested with NheI and HindIII to make pcDNA3aS.

Gel Electrophoresis and Western Blotting. Immune pellets were resolved on 12% SDS−polyacrylamide gels and transferred to Immobilon-P membranes. The membranes were probed with 5 μg/mL anti-CYP2E1, 1 μg/mL AC88 for hsp90, 1 μg/mL N27F3-4 for hsp70, 0.5 μg/mL anti-CHIP, or 0.5% anti-ubiquitin. The immunoblots were then incubated a second time with the appropriate ^{125}I-conjugated or horseradish peroxidase-conjugated counter antibody to visualize the immunoreactive bands. Experiments were repeated two to four times, and a single Western blot is presented.

RESULTS

Treatment with Radicicol Increases the Extent of Proteasomal Degradation of CYP2E1. Because the stability of a large number of proteins that are degraded by the ubiquitin−proteasome pathway is determined by assembly of heterocomplexes with hsp90 (*10*), we asked if the hsp90 inhibitor radicicol would promote the degradation of CYP2E1. hsp90 is a member of a very limited family of proteins, the GHKL family, which possess a unique binding pocket for ATP (*33*). The hsp90 inhibitors geldanamycin and radicicol bind to this nucleotide site (*34*, *35*) and prevent hsp90 from achieving its ATP-dependent conformation, thus blocking hsp90 action (*36*). In the experiments depicted in Figure 1, transformed human fibroblasts stably expressing CYP2E1 (*23*) were treated with radicicol. Inhibition of hsp90 function by radicicol yielded a concentration-dependent (Figure 1A) and time-dependent (Figure 1B) decrease in CYP2E1 activity. As shown in Figure 1C, the amount of CYP2E1 protein decreased in radicicol-treated cells, and the decrease was attenuated by simultaneous exposure to lactacystin. These data suggest that CYP2E1 is stabilized by hsp90. Like other client proteins, CYP2E1 is degraded by the ubiquitin−proteasome pathway when hsp90 heterocomplex assembly is inhibited.

Because CYP2E1 is a microsomal enzyme and we require a soluble form of the enzyme for immunoadsorption, we could not determine if the full-length CYP2E1 was in

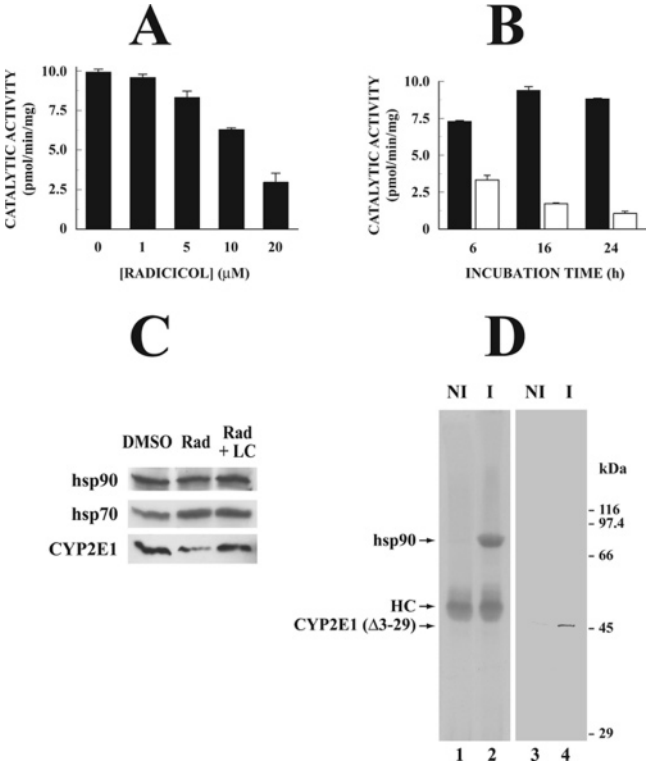

FIGURE 1: Evidence that CYP2E1 turnover is regulated by hsp90. GM2E1 cells were treated with radicicol, and 7-ethoxycoumarin *O*-deethylase activity was assayed in intact cells. (A) Concentration dependence of the 16 h radicicol treatment. (B) Time course of 20 μM radicicol inhibition: radicicol-treated (white bars) and DMSO (black bars). The data in panels A and B represent the mean ± the standard error of the mean for three experiments. (C) Radicicol-dependent increase in the extent of CYP2E1 degradation. GM2E1 cells were treated for 6 h with DMSO vehicle, 20 μM radicicol (Rad), or radicicol with 10 μM lactacystin (LC). Cells were then sonicated and boiled in SDS sample buffer, and CYP2E1, hsp90, and hsp70 were resolved by SDS−polyacrylamide gel electrophoresis and immunoblotting. (D) CYP2E1(Δ3−29) is in complexes with hsp90. HEK293 cells were transiently transfected with the Δ3−29 N-terminal truncation of CYP2E1, and after 48 h, cytosol was prepared and immunoadsorbed with nonimmune IgM (NI) or 8D3 monoclonal IgM against hsp90 (I). The immunopellets were washed, and proteins were resolved by electrophoresis and immunoblotting. Lanes 1 and 2 were blotted for hsp90; lanes 3 and 4 were blotted for CYP2E1. HC stands for the heavy chain of the mouse IgG against IgM, which reacts with the anti-mouse counter antibody in lanes 1 and 2 but not with the anti-rabbit counter antibody in lanes 3 and 4.

heterocomplexes with hsp90. For this purpose, we expressed a truncated form of rabbit CYP2E1 lacking the hydrophobic N-terminal signal peptide (*24*). This truncation, CYP2E1-(Δ3−29), is fully catalytically active when the bacterially expressed enzyme is reconstituted with lipid and reductase (*24*). When it is expressed in HEK293T cells, ∼10% of the enzyme is in the cytosolic fraction. Because antibodies against CYP2E1 do not immunoadsorb the enzyme, to determine if heterocomplexes exist, we immunoadsorbed hsp90 from cytosol of 293T cells expressing CYP2E1(Δ3−29) and immunoblotted the hsp90 immune pellets for co-immunoadsorbed P450. As shown in Figure 1D, CYP2E1-(Δ3−29) co-immunoadsorbs with hsp90, an observation that suggests that CYP2E1·hsp90 heterocomplexes form in vivo.

Effect of CHIP Expression in HEK293T Cells. In the experiment depicted in Figure 2A, CHIP and CYP2E1 were coexpressed in HEK293T cells and the level of each protein

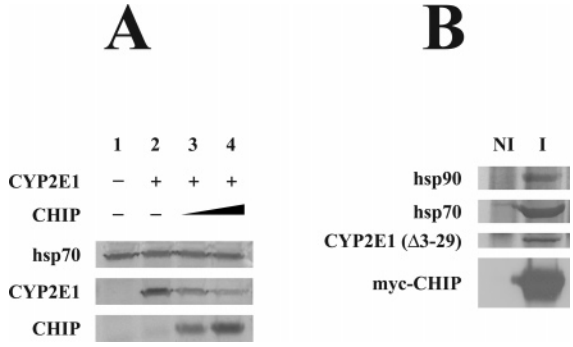

FIGURE 2: CHIP promotes CYP2E1 degradation in HEK293 cells. (A) CHIP promotes CYP2E1 degradation. HEK293 cells were cotransfected with a plasmid expressing CYP2E1 and either control plasmid or 2 or 5 μg of plasmid expressing CHIP. Forty-eight hours later, cell lysates were prepared and immunoblotted for CYP2E1, hsp70, and CHIP. (B) CHIP is found in heterocomplexes with CYP2E1(Δ3−29) and hsp70. HEK293 cells were transfected for 48 h with myc-CHIP and CYP2E1(Δ3−29), and cytosol was prepared and immunoadsorbed with nonimmune (NI) or anti-myc (I) antibody followed by Western blot analysis with anti-CYP2E1, anti-hsp90, anti-hsp70, and anti-CHIP antibodies.

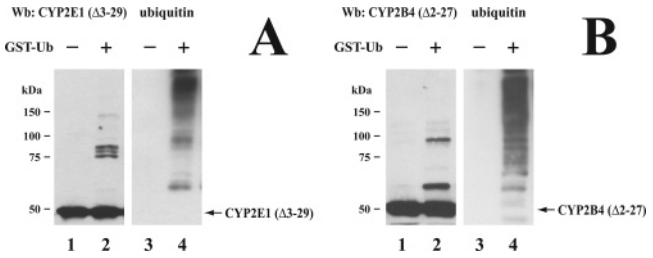

FIGURE 3: P450 ubiquitylation in an in vitro system comprised of purified ubiquitylating enzymes. Purified CYP2E1(Δ3−29) (A) or CYP2B4(Δ2−27) (B) was incubated with a reaction mixture containing purified E1 ubiquitin activating enzyme, E2-conjugating enzyme, and the CHIP E3 ligase in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of GST−Ub as described in Materials and Methods. The ubiquitylated products were resolved by SDS−polyacrylamide gel electrophoresis and immunoblotting with antibody against P450 (lanes 1 and 2) or ubiquitin (lanes 3 and 4).

was assessed by immunoblotting whole cell lysates 48 h after transfection. It can be seen that expression of CHIP results in a lower level of CYP2E1, suggesting that CHIP can promote CYP2E1 degradation. To determine if CHIP was in complexes with P450, we cotransfected cells with CYP2E1(Δ3−29) and myc-CHIP. After 48 h, cytosol was prepared and myc-CHIP was immunoadsorbed with the anti-myc antibody. As shown in Figure 2B, some CYP2E1(Δ3−29) was co-immunoadsorbed with myc-CHIP, as was a large amount of hsp70 and some hsp90. This suggests that CYP2E1 and CHIP can exist in the same heterocomplexes in vivo.

Ubiquitylation of Purified CYP2E1(Δ3−29). To study CHIP-dependent ubiquitylation, we set up a cell-free system in which purified, bacterially expressed CYP2E1(Δ3−29) was incubated with a purified ubiquitylating system consisting of an E1 ubiquitin-activating enzyme, an E2 ubiquitin carrier protein conjugating enzyme (UbcH5a), CHIP, GST−Ub, and an ATP-generating system. As shown in Figure 3A in the left panel, CYP2E1(Δ3−29) is converted to higher-molecular mass bands that immunoblot with anti-CYP2E1 (lane 2), and in the right panel, incubation with GST−Ub yields multiple high-molecular mass species that immunoblot with anti-Ub (lane 4). Figure 3B shows that purified

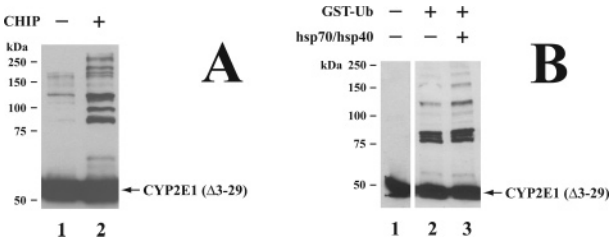

FIGURE 4: CYP2E1 ubiquitylation in vitro is CHIP-dependent. (A) Ubiquitylation is CHIP-dependent. Purified CYP2E1(Δ3−29) was incubated with E1, E2, and GST−Ub in the absence (−) or presence (+) of CHIP and Western blotted with anti-CYP2E1. (B) Hsp70 increases the extent of CYP2E1 ubiquitylation. Purified CYP2E1-(Δ3−29) was preincubated for 5 min at 30 °C with hsp70, hsp40, and an ATP-generating system. Samples were then incubated for 30 min with purified E1, E2, and CHIP in the presence or absence of GST−Ub. Samples were Western blotted with anti-CYP2E1.

CYP2B4(Δ2−27) can also be ubiquitylated by the same system.

The production of GST−Ub conjugates of CYP2E1(Δ3−29) is highly dependent upon the presence of CHIP in the ubiquitylation mix (Figure 4A). The RING-type E3 enzymes, like CHIP, are thought to act as bridging proteins that bring the ubiquitin-charged E2 enzyme into the vicinity of the substrate (*37*). However, it is not known if CHIP itself contacts the substrate, and it is thought that the chaperones target CHIP to the protein that is to be ubiquitylated (*22*, *38*). However, preincubation of CYP2E1(Δ3−27) with hsp70 and hsp40 prior to incubation with CHIP and the rest of the ubiquitylating system yielded only a small increase in the amount of high-molecular mass products (Figure 4B, cf. lanes 2 and 3). Western blotting of the commercial preparation of E1 revealed considerable contamination with rabbit hsp70 (data not shown), and the failure of added hsp70 to substantially increase the level of CHIP-dependent ubiquitylation may reflect this presence of hsp70 in the ubiquitylation mix.

CCl_4-Mediated Degradation of CYP2E1. The effect of treating HEK293T cells expressing CYP2E1 with CCl_4 is shown in Figure 5. Treatment with CCl_4 causes a rapid and selective (see no change in hsp90 and hsp70) loss of immunodetectable CYP2E1 (Figure 5A) that is concentration-dependent (Figure 5B). The CCl_4-dependent loss of CYP2E1 is inhibited by treatment with MG132 (Figure 5C), showing that degradation is via the proteasomal pathway. If CCl_4-mediated degradation is dependent upon binding of hsp70 to the damaged enzyme, then one might find an increase in the amount of CYP2E1(Δ3−29) being coadsorbed with hsp70 from cytosol of transfected cells treated with CCl_4. As shown in Figure 5D, immunoadsorption of hsp70 yields co-immunoadsorption of CYP2E1(Δ3−29), suggesting that the enzyme and hsp70 can exist in the same complexes. Unfortunately, we found that CYP2E1(Δ3−29) was not degraded upon treatment with concentrations of CCl_4 up to 10 times the concentration of the suicide substrate that leads to the proteasomal degradation of full-length CYP2E1 (Figure 5E). The absence of CCl_4 effect may reflect a failure of the expressed CYP2E1(Δ3−29) to link appropriately with endogenous, membrane-bound reductase.

Reductase-Dependent and CCl_4-Dependent Ubiquitylation of Purified CYP2E1(Δ3−29). Purified CYP2E1(Δ3−29) can be mixed with purified P450 reductase and lipid to yield a

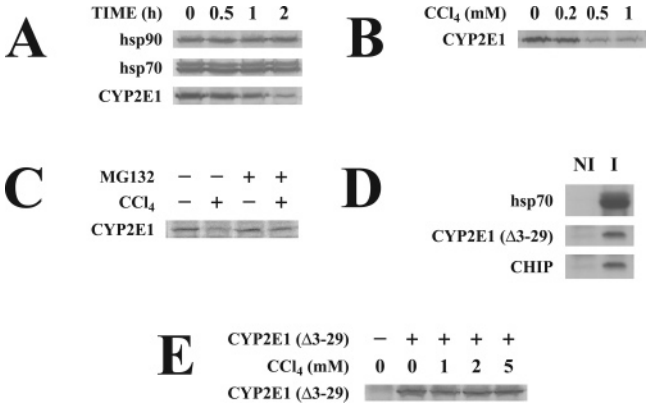

FIGURE 5: Wild-type CYP2E1 is selectively degraded when cells are treated with CCl_4, but CYP2E1(Δ3−29) is not. (A) Time course of degradation. HEK293T cells expressing CYP2E1 were treated for the indicated times with 0.5 mM CCl_4, and aliquots of cell lysates were resolved by SDS−polyacrylamide gel electrophoresis and immunoblotting for CYP2E1, hsp90, and hsp70. (B) Concentration dependence of the CCl_4 effect. CYP2E1-expressing cells were treated for 3 h with the indicated concentrations of CCl_4. (C) CCl_4-induced degradation is inhibited by MG132. CYP2E1-expressing cells were treated (+) for 1 h or not (−) with 0.5 mM CCl_4 and/or 10 μM MG132 as indicated, and aliquots of whole cell lysates were immunoblotted for CYP2E1. (D) CYP2E1(Δ3−29) is co-immunoadsorbed with hsp70. hsp70 was immunoadsorbed with nonimmune IgG (NI) or with 10 μL of BB70 ascites fluid (I) from 100 μL of cytosol prepared from cells expressing CYP2E1-(Δ3−29), and the washed immune pellets were immunoblotted for hsp70, CHIP, and CYP2E1(Δ3−29). (E) Cells expressing CYP2E1-(Δ3−29) were treated with various concentrations of CCl_4.

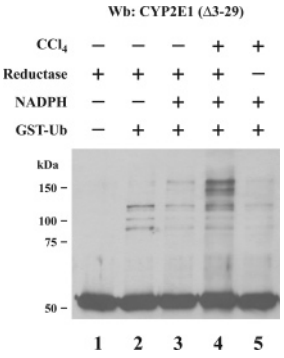

FIGURE 6: CCl_4 stimulates CYP2E1(Δ3−29) ubiquitylation in a reductase-coupled in vitro system. Purified CYP2E1(Δ3−29) was preincubated with phosphatidylcholine in the absence (−) or presence (+) of P450 reductase, NADPH, and CCl_4 as described in Materials and Methods. An aliquot of this preincubated mix was then incubated with purified E1, E2, and CHIP in the presence or absence of GST−Ub. Samples were Western blotted with anti-CYP2E1.

catalytically active enzyme (*24*). In the experiment depicted in Figure 6, the catalytically active mixture was prepared and incubated with CCl_4 prior to incubation with the in vitro ubiquitylation mixture. Under these conditions, CCl_4 treatment caused a marked increase in the extent of CYP2E1-(Δ3−29) ubiquitylation (cf. lanes 3 and 4) that is dependent upon the presence of reductase (cf. lanes 4 and 5).

DISCUSSION

We have shown here that treatment of GM2E1 cells with the hsp90-specific inhibitor radicicol results in the loss of CYP2E1 activity and CYP2E1 protein, with the loss of protein being inhibited by lactacystin (Figure 1). This is

consistent with a model in which the hsp90/hsp70-based chaperone machinery is responsible for hsp90-dependent stabilization of CYP2E1 from proteolysis via the ubiquitin−proteasome pathway. This observation stands in contrast to the results of Huan et al. (*6*) who found no effect of geldanamycin or radicicol on CYP2E1 turnover in HeLa cells where expression of the P450 was under the control of tetracycline. Goasduff and Cederbaum (*5*) examined the effect of geldanamycin in an in vitro system containing human liver microsomes where degradation of CYP2E1 required the addition of cytosol and was inhibited by proteasome inhibitors. They found that geldanamycin inhibited the degradation of microsomal CYP2E1 by the cytosol. Also, immunodepletion of hsp90 from cytosol prevented CYP2E1 degradation, and degradation was restored by purified hsp90, leading the authors to conclude that hsp90 promotes the proteasomal degradation of CYP2E1 (*5*). This conclusion stands in contrast to the model established for all other hsp90 client proteins (*10*) in which formation of a complex with hsp90 stabilizes them against proteasomal degradation.

Geldanamycin has been found to inhibit hemin-mediated restoration of CYP2B1 after inactivation by allylisopropylacetamide, a suicide inactivator that destroys the heme by N-alkylation of the pyrrole ring (*39*). The geldanamycin inhibition is ascribed to an effect on Grp94, which is required for hemin-mediated reassembly of heme-stripped microsomal CYP2B1 (*39*). Grp94 is a member of the hsp90 family that is localized to the endoplasmic reticulum, and like hsp90, it is inhibited by geldanamycin and radicicol. It is interesting that geldanamycin and radicicol also inhibit heme activation of apo-nNOS (*12*, *40*), and in the case of nNOS, there is direct evidence that assembly of complexes with hsp90 facilitates opening of the hydrophobic heme binding cleft to access by heme (*41*). Similarly, assembly of complexes with hsp90 is required for the hydrophobic ligand binding clefts of glucocorticoid, mineralocorticoid, and aryl hydrocarbon receptors to be open and accessible to their ligands (*11*).

The first drug metabolizing enzyme shown to undergo degradation upon geldanamycin treatment was cytosolic thiopurine *S*-methyltransferase (TPMT), which catalyzes the S-methylation of thiopurine drugs such as 6-mercaptopurine (*42*). TPMT is assembled into complexes with hsp90 by reticulocyte lysate, and when COS-1 cells expressing TPMT are treated with geldanamycin, TPMT is subject to rapid proteolysis via the ubiquitin−proteasome pathway (*42*). Some genetic polymorphisms in TPMT are associated with greatly increased drug toxicity. The most common variant in Caucasians results in low TPMT protein levels because of rapid degradation via the ubiquitin−proteasome pathway (*42*). It is reasonable to predict that pharmacogenetic variants in CYP2E1 and probably other P450s will be found to reflect alterations in the dynamics of hsp90 heterocomplex assembly, leading to decreased protein levels and altered drug response.

Because TPMT is a cytosolic drug metabolizing enzyme, direct studies of hsp90 binding and regulation can be performed on the full-length, catalytically active enzyme. Our study of hsp90 regulation of CYP2E1 is presented, however, with the caveat that we make conclusions regarding the behavior of the full-length, membrane-bound, and catalytically active form of CYP2E1 while co-immunoprecipitation data are obtained with the cytosolic portion of the truncated CYP2E1(Δ3−29) and ubiquitylation data are obtained with the bacterially expressed truncated enzyme. The harsh detergent conditions required to solubilize full-length CYP2E1 from membrane destroy chaperone and CHIP binding, and our study is further compromised by the fact that we do not have a means of directly immunoadsorbing the enzyme. Thus, we show that inhibition of hsp90 with radicicol (Figure 1C) and overexpression of CHIP (Figure 2A) promote degradation of full-length CYP2E1 in GM2E1 and HEK293 cells, respectively, but binding to hsp90 and myc-CHIP is shown by co-immunoprecipitation of the truncated CYP2E1-(Δ3−29) with the associated protein from transfected HEK cell cytosol. As the CYP2E1(Δ3−29) is catalytically inactive in the cell, it is fair to ask whether binding of hsp90 or CHIP with the truncated enzyme reflects what is occurring with the catalytically active, full-length, membrane-bound enzyme. Because the truncated enzyme can be coupled with P450 reductase to yield a catalytically active enzyme (ref *24* and Figure 6), we would argue that its interactions with hsp90, hsp70, and CHIP may reflect what is occurring with the membrane-bound form of the enzyme that interacts with the reductase.

Although there is broad agreement that CYP2E1 undergoes proteasomal degradation (*2*−*8*), there is controversy about whether it is ubiquitylated (*2*, *4*, *8*) or not (*3*, *6*), and Huan et al. (*6*) have suggested that the enzyme is degraded by the proteasome by a ubiquitin-independent pathway. Here we show that the ubiquitin E3 ligase CHIP lowers the level of CYP2E1 and that CYP2E1(Δ3−29) exists in heterocomplexes with CHIP (Figure 2). Also, cell-free ubiquitylation of CYP2E1(Δ3−29) by a ubiquitylating enzyme mix is CHIP-dependent (Figure 4A).

There are several known ways in which proteins are recognized for ubiquitylation, such as by N-end rule, allosteric regulation, phosphorylation, etc. (reviewed in ref *43*), but the method by which the cell surveils for damaged proteins or non-native proteins to target them for ubiquitylation is not known (*44*). The signal is thought to be the exposure of hydrophobic domains that are normally buried within the protein core (*43*). A site where this exposure should occur at the earliest stages of unfolding is where hydrophobic clefts open to fuse with the surface of the protein. Two components of the chaperone machinery, hsp70 and hsp40, have been shown to be essential for the ubiquitylation and degradation of some abnormal proteins (*45*, *46*). We know from mechanistic studies of GR·hsp90 heterocomplex assembly that the first step is interaction of the GR with the hsp70 component of the assembly machinery (*47*, *48*), and we found with nNOS (*15*) that interaction of CHIP with substrate-bound hsp70 appears to be required for the ubiquitylation that targets proteasomal degradation. Thus, it is reasonable to propose that hsp70 functions as a sensor for protein triage by detecting the native versus early denaturing states of proteins.

To begin to examine this possibility, we used CCl_4, which acts as a mechanism-based inactivator to cross-link CYP2E1 to heme within the hydrophobic heme binding cleft (*49*), triggering ubiquitylation and selective loss of the enzyme (*4*). Using CCl_4 as a site-specific means of causing cleft damage, we repeated the observations of others that CCl_4 treatment causes selective degradation of CYP2E1 via the

proteasomal pathway (Figure 5A−C). However, treatment of cells expressing CYP2E1(Δ3−29) with very high concentrations of CCl_4 did not lead to loss of immunodetectable enzyme (Figure 5E). Thus, although CYP2E1(Δ3−29) co-immunoadsorbs with hsp70 (Figure 5D), we do not have a way to determine if degradation of the truncated enzyme in the intact cell is hsp70-dependent. However, it is a reasonable proposal that hsp70 is the sensor for protein triage between hsp90 heterocomplex assembly with preservation of protein function and CHIP-dependent ubiquitylation leading to proteasomal degradation.

The turnover of CYP2E1 is variable and complex [see Liao et al. (*50*) and Correia et al. (*51*) for discussion and references]. The turnover can be modified by substrate, but how substrate effects on the heme−substrate binding cleft in the enzyme may affect the interaction of CYP2E1 with the hsp90/hsp70-based chaperone machinery to modify turnover is uncertain. In mammalian liver, CYP2E1 has biphasic turnover, with a rapid phase $t_{1/2}$ of ∼7 h and a slow phase $t_{1/2}$ of ∼37 h. In the presence of ethanol, the half-life is long (∼37 h) and the enzyme is degraded by the lysosomal pathway. This may be analogous to the steroid receptors where the presence of steroid in the hydrophobic ligand binding cleft favors a closed cleft conformation that no longer interacts with the chaperone machinery to form stable receptor heterocomplexes (*11*). The presence of ethanol in the heme−substrate cleft of the enzyme may favor a closed state of the hydrophobic cleft that does not interact with hsp70 to permit CHIP-dependent ubiquitylation. When ethanol is withdrawn, CYP2E1 degradation is rapid (∼7 h) and degradation is inhibited by proteasome inhibitors. In the absence of ethanol, CYP2E1 can interact with the chaperone machinery, the enzyme is stabilized by hsp90, and treatment with an hsp90 inhibitor yields even more rapid degradation via the proteasomal pathway (Figure 1). In the presence of the mechanism-based inactivator CCl_4, cleft damage may result in exposure of the hydrophobic cleft interior that interacts with a conformation of hsp70 favoring CHIP binding, resulting in even more rapid ubiquitylation and proteasomal degradation.

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