CYP2C9-CYP3A4 Protein-Protein Interactions: Role of the Hydrophobic N Terminus

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ABSTRACT:

Cytochromes P450 (P450s) interact with redox transfer proteins, including P450 reductase (CPR) and cytochrome b_5 (b_5), all being membrane-bound. In multiple in vitro systems, P450-P450 interactions also have been observed, resulting in alterations in enzymatic activity. The current work investigated the effects and mechanisms of interaction between CYP2C9 and CYP3A4 in a reconstituted system. CYP2C9-mediated metabolism of S-naproxen and S-flurbiprofen was inhibited up to 80% by coinubation with CYP3A4, although K_m values were unchanged. Increasing CYP3A4 concentrations increased the degree of inhibition, whereas increasing CPR concentrations resulted in less inhibition. Addition of b_5 only marginally affected the magnitude of inhibition. In contrast,

CYP2C9 did not alter the CYP3A4-mediated metabolism of testosterone. The potential role of the hydrophobic N terminus on these interactions was assessed by incubating truncated CYP2C9 with full-length CYP3A4, and vice versa. In both cases, the inhibition was fully abolished, indicating an important role for hydrophobic forces in CYP2C9-CYP3A4 interactions. Finally, a CYP2C9/CYP3A4 heteromer complex was isolated by coimmunoprecipitation techniques, confirming the physical interaction of the proteins. These results show that the N-terminal membrane binding domains of CYP2C9 and CYP3A4 are involved in heteromer complex formation and that at least one consequence is a reduction in CYP2C9 activity.

Cytochromes P450 (P450s) are the primary oxidative enzymes responsible for the metabolism of xenobiotics by humans and animals (Guengerich, 2006). P450s can oxidize structurally diverse substrates ranging from small hydrocarbons to large molecules such as cyclosporine. P450s function by transferring electrons via a P450 catalytic pathway, wherein the first electron is transferred to the P450 via cytochrome NADPH P450 reductase (CPR) and the second electron is transferred via either CPR or cytochrome b_5 (b_5) (Guengerich, 2002). Inefficiencies in the catalytic pathway, in part, govern the rate of transformation. P450s and CPR are bound to the cell membrane via a hydrophobic N terminus, whereas b_5 is bound via its C terminus, each terminal being composed of 30 to 60 amino acids (Szczesna-Skorupa and Kemper, 2008). This hydrophobic region also contributes to protein aggregation, resulting in the formation of high molecular weight polymers in membranes and reconstituted systems (Szczesna-Skorupa and Kemper, 2008). Considering that P450s and CPR, and P450s and b_5 interact within the cell membrane, P450-P450 interactions also appear conceivable. Indeed, in vitro evidence of P450-P450 interactions have been observed in several studies, including interactions of human CYP2C9-CYP2C19, rabbit CYP1A2-CYP2B4, rabbit CYP1A2-CYP2E1, human CYP3A4-CYP1A1, human CYP3A4-CYP1A2, and human CYP2C9-CYP2D6 (Cawley

et al., 1995; Yamazaki et al., 1997; Backes et al., 1998; Backes and Cawley, 1999; Hazai and Kupfer, 2005; Kelley et al., 2005, 2006; Subramanian et al., 2009). Substrate-dependent activation or inhibition of one or both isoforms has been observed. These P450-P450 interactions have also been observed in triple-expression systems and microsomes isolated from rabbits after administration of a selective inducer (Kaminsky and Guengerich, 1985; Tan et al., 1997; Li et al., 1999; Cawley et al., 2001).

Direct physical evidence of interactions between P450s, defined as the formation of heteromers, has been more challenging to establish mainly because of their lipophilic nature and the tendency to aggregate. CYP1A1-CYP3A2 and CYP2E1-CYP2C2 complexes were identified using bimolecular fluorescence complementation spectroscopy and chemical cross-linking followed by immunoprecipitation, respectively (Alston et al., 1991; Ozalp et al., 2005). In addition, molecular modeling studies have also suggested the formation of heteromers (Backes and Kelley, 2003; Hazai et al., 2005), whereas an order of addition study provided indirect evidence for the formation of quasi-irreversibly formed CYP2C9 and CYP2D6 heteromers (Subramanian et al., 2009).

Although the in vitro evidence for P450-P450 interactions is compelling, the in vivo evidence of and physiological significance for P450-P450 interactions remains to be established. It has been speculated that heteromerization of P450s may exist to ensure proper cellular targeting into the membrane, cytosol, and Golgi complexes (Szczesna-Skorupa and Kemper, 2008). Another study showed that CYP3A4 catalyzed the oxidation and peroxidation of microsomal

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ABBREVIATIONS: P450, cytochrome P450; CPR, cytochrome P450 reductase; b_5 , cytochrome b_5 ; DOPC, L- α -dioleoyl-*sn*-glycero-3-phosphocholine; TBST, Tris-buffered saline/Tween; DHT, dihydrotestosterone; TST, testosterone; rCPR, rat cytochrome P450 reductase; hCPR, human cytochrome P450 reductase; CYP2C9(t), truncated CYP2C9; CYP3A4(t), truncated CYP3A4.

lipids in the absence of substrates, thereby making the cells susceptible to oxidative damage (Kimzey et al., 2003). These hydroperoxides, in turn, govern the homomerization and heteromerization of CYP3A4, thereby reducing CYP3A4-mediated lipid metabolism through product inhibition, and hence reduce the risk of oxidative damage. In the presence of substrates, this polymerization mechanism may preserve the catalytically significant P450 isoforms and facilitate the aggregation of P450 isoforms that lack substrate, thereby decreasing their levels (Kimzey et al., 2003). It has been reported that P450s are nonuniformly distributed in the endoplasmic reticulum, forming clusters rather than possessing a continuous distribution (Matsuura et al., 1978), whereas other reviews have suggested the existence of zonal differences in P450 distribution within the liver (Jungermann, 1995; Oinonen and Lindros, 1998). These differences in distribution would be expected to promote P450-P450 interactions.

Numerous factors have been suggested as potential mechanisms for the observed changes in activity as a result of P450-P450 interactions. Competition for CPR, ionic interactions, conformational changes in the P450, obstruction of the substrate binding site, obstruction of the CPR binding site, and the formation of heteromers with altered activity from monomers also have been speculated as potential mechanisms governing P450-P450 interactions (Backes and Kelley, 2003; Hazai et al., 2005). The present work assesses the interaction of two major P450 isoforms (CYP2C9 and CYP3A4), including direct evidence of oligomerization, the effect on enzyme activity, and the role of the hydrophobic N terminus in these interactions.

Materials and Methods

Materials. Acetonitrile, dibasic potassium phosphate, methanol, phosphoric acid, and terrific broth were purchased from Thermo Fisher Scientific (Waltham, MA). Dilauroylphosphatidylcholine, NADPH, formic acid, protease inhibitors, DNAase, lysozyme, sodium cholate, Tergitol, ampicillin, isopropyl β -D-1-thiogalactopyranoside, thiamine, imidazole, β -mercaptoethanol, EDTA, potassium phosphate, L- α -dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), CHAPS, Tris, Tris-buffered saline, and Tris-buffered saline/Tween (TBST) were purchased from Sigma-Aldrich (St. Louis, MO). (*S*)-Naproxen and desmethylnaproxen were gifts from the former Syntex Labs (Palo Alto, CA). Dihydrotestosterone (DHT), testosterone (TST), and 6- β -hydroxytestosterone were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Hydroxyapatite was purchased from Bio-Rad Laboratories (Hercules, CA). (*S*)-Flurbiprofen, 4'-hydroxyflurbiprofen, and 2-fluoro-4-biphenyl acetic acid were gifts from the former Pharmacia, Inc. (Kalamazoo, MI). Human CPR and b5 were purchased from Invitrogen (Carlsbad, CA). CYP2C9.1 and truncated CYP2C9.1 were expressed and purified as described previously (Locuson et al., 2006). Western blotting supplies, bacterial protein extraction reagent, and a protein coimmunoprecipitation kit with gentle binding and elution buffers were purchased from Pierce Chemical (Rockford, IL). CYP2C9 and CYP3A4 antibodies were purchased from BD Biosciences (San Jose, CA), and the goat anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). CYP3A4 plasmid and truncated CYP3A4 were gifts from Dr. William Atkins (University of Washington, Seattle, WA), and rat reductase (rCPR) was a gift from Professor Paul Hollenberg (University of Michigan, Ann Arbor, MI).

Expression and Purification of CYP3A4. CYP3A4 was expressed and purified in a manner similar to the preparation of CYP2C9, as described previously (Locuson et al., 2006). In brief, *Escherichia coli* transformed with the CYP3A4 construct in a pCW vector, with ampicillin resistance and a 6 $\times$ histidine tag, was streaked on a Luria broth agar plate. Single colonies were added to 10 ml of Luria broth with ampicillin and grown overnight at 37°C and at 225 rpm. These primary cultures were used to incubate 0.5-liter terrific broth cultures containing trace elements, ampicillin, and thiamine. On reaching an optical density of 0.4 to 0.6, usually in approximately 4 h, the 0.5-liter cultures were induced with isopropyl β -D-1-thiogalactopyranoside, vitamins, and alanine supplements. The induced cultures were then maintained at a lower

temperature and speed of 25°C and 175 rpm, respectively. The cultures were harvested 16 h postinduction, and the pellets were stored at -80°C until purification. For purification of CYP3A4, the pellets were solubilized at 4°C in bacterial protein extraction reagent, sodium chloride, detergent (sodium cholate and Tergitol), EDTA, imidazole, protease inhibitors, lysozyme, DNAase and β -mercaptoethanol, and the protein then was extracted using a French press. After ultracentrifugation at 100,000g, the protein from the supernatant was purified using a nickel column. Hydroxyapatite resin was then used to eliminate all the detergent, and the resulting protein was dialyzed overnight twice to further purify the protein. Finally, the enzyme was concentrated using a Centricon (Millipore Corporation, Billerica, MA) centrifuge concentrator with a molecular mass cutoff of 12 kDa. Purity was assessed by SDS-polyacrylamide gel electrophoresis, and a single band at 57 kDa was detected.

(*S*)-Flurbiprofen and (*S*)-Naproxen Metabolism by CYP2C9 in the Presence of CYP3A4. Multiple concentrations of (*S*)-flurbiprofen from 2 to 300 μ M were incubated with CYP2C9 (0.025 μ M), CPR (0.05 or 0.1 μ M for subsaturating or saturating conditions, respectively), and CYP3A4 (0, 0.025, or 0.05 μ M). CYP2C9 was mixed with CYP3A4, allowed to equilibrate on ice for 5 min, after which CPR was added and the ternary mixture allowed to further equilibrate on ice for 5 min. The mixture next was reconstituted in dilauroylphosphatidylcholine (extruded through a 200-nm pore size membrane) and allowed to equilibrate for 5 min. Enzyme mixtures were then added to substrate and buffer and preincubated at 37°C for 5 min before initiation of the reaction. All the experiments were carried out in 50 mM potassium phosphate buffer, pH 7.4, at 37°C. After initiation of the reaction with NADPH (1 mM final concentration), the reactions were allowed to continue for 20 min and then terminated by the addition of 200 μ l of 180 ng/ml 2-fluoro-4-biphenyl acetic acid (internal standard) in acetonitrile. To the quenched reaction was then added 40 μ l of half-strength phosphoric acid to adjust the pH to $\sim$ 3.0. All the experiments were repeated three times on separate days. The amount of 4'-OH-flurbiprofen produced was used as a measure of CYP2C9 activity. Similar experiments were repeated with (*S*)-naproxen as a substrate (10–1800 μ M naproxen concentration range). Likewise, the amount of desmethyl-naproxen produced was also used as a measure of CYP2C9 activity.

The incubations using flurbiprofen as substrate were also repeated with rCPR instead of human P450 reductase (hCPR) using the same incubation conditions. Because the results were identical, subsequent incubations were conducted with rCPR. The effect of b5 on CYP2C9-CYP3A4 interactions also were tested with flurbiprofen as a substrate using a ratio of 1:1:2:1 CYP2C9/CYP3A4/rCPR/b5.

CYP2C9-CYP3A4 interaction incubations were also performed wherein one or both of the isoforms were truncated (i.e., minus the N terminus). The truncated CYP2C9 and CYP3A4 isoforms [CYP2C9(t) and CYP3A4(t), respectively] were missing their first 30 amino acids comprising the N terminus membrane binding hydrophobic domain. The CYP2C9(t)-CYP3A4 and CYP2C9-CYP3A4(t) incubations were performed at 1:1:2 of CYP2C9/CYP3A4/rCPR with flurbiprofen as a substrate, according to the previously described incubation conditions.

TST Metabolism by CYP3A4 in the Presence of CYP2C9. The order of reconstitution has been shown to be an important determinant in CYP3A4 incubations, and hence a previously established protocol was followed (Yamazaki et al., 1997). Tris, CHAPS, DOPC, CPR, b5, CYP3A4, and CYP2C9 (if present) were mixed in that order, and a 5-min equilibration on ice was allowed after addition of each protein component. Final concentrations were 50 mM TRIS, 0.4 μ g/ml CHAPS, 100 μ g/ml DOPC, 0.2 μ M CPR, 0.1 μ M b5, 0.05 μ M CYP3A4, and 0.05 μ M CYP2C9, respectively. The 2-mg/ml original stock DOPC solution was filtered through a 200-nm extruder membrane. The substrate, TST, was added to the aliquoted enzyme mixture, and after 3 min of preincubation at 37°C, the reaction was initiated with NADPH (1 mM final concentration). The reaction was quenched at 7.5 min by the addition of 100 μ l of acetonitrile containing 700 ng/ml DHT as an internal standard. Linearity of TST metabolism to its 6-hydroxy metabolite as catalyzed by CYP3A4 was evaluated with respect to time and protein concentration. The influence of CYP2C9 on CYP3A4 metabolism was tested at TST concentrations ranging from 25 to 500 μ M. Again, all the experiments were performed three times on separate days.

Analysis of 4'-Hydroxyflurbiprofen and Desmethylnaproxen. Quantitation of 4'-hydroxyflurbiprofen and desmethylnaproxen formation was carried out exactly as described previously (Tracy et al., 1997, 2002).

Analysis of 6- β -Hydroxytestosterone. A Brownlee Spheri-5 C18 column (PerkinElmer Life and Analytical Sciences, Waltham, MA), 100 $\times$ 4.6 mm, 5- μ m particle size was used for the separation and quantification of TST and its metabolites. The mobile phase consisted of 90% 10 mM ammonium formate/10% methanol (A) and 100% methanol (B) delivered via an Agilent 1100 (Agilent Technologies, Santa Clara, CA) autosampler and pumps to an ABI SCIEX liquid chromatography/tandem mass spectrometry QTRAP 2000 mass spectrometer (Applied Biosystems, Foster City, CA). The mass spectrometer was operated in multiple-reaction monitoring, positive mode to detect 6- β -hydroxytestosterone (305–269 m/z transition), TST (289–109 m/z), and the internal standard DHT (291–255 m/z), which eluted at 6.8, 10.6, and 12.2 min, respectively. For the mass spectrometer, the curtain gas (nitrogen) was maintained at 30 psi, collision gas at 12 psi, ion spray voltage at 5500 V, temperature at 400°C, ion source gas 1 at 70 psi, ion source gas 2 at 60 psi, declustering potential at 55 V, entrance potential at 11 V, collision energy at 25 V, and collision cell exit potential at 3 V. The liquid chromatography method involved a gradient run at 500 μ l/min with the following compositions: 72.5% A 0 to 2 min, 72.5 to 54% A 2 to 2.5 min, maintained at 54% A until 7.5 min, 54 to 25% A from 7.5 to 8 min, maintained at 24% A until 13 min, 24 to 72.5% A from 13 to 13.5 min, and a re-equilibration step of 72.5% A from 13.5 to 15 min.

Coimmunoprecipitation of CYP2C9 and CYP3A4. A Pierce Chemical coimmunoprecipitation kit was used, according to the manufacturer's protocol, to evaluate the formation of CYP2C9-CYP3A4 heteromers. Coimmunoprecipitation was also attempted with CYP2C9 and CYP3A4(t). In brief, 100 μ l of coupling resin slurry (50% solution) was washed with gentle binding buffer twice and incubated with anti-CYP2C9 or anti-CYP3A4 (30 μ l of antibody plus 120 μ l of binding buffer, $\sim$ 100 μ g of antibody) and sodium cyanoborohydride overnight at room temperature. The resin was washed with binding buffer and next quenched with quenching buffer and sodium cyanoborohydride for 30 min. It was subsequently washed with wash buffer and then binding buffer. To pull down the CYP2C9-CYP3A4 complex, separate resin vials bound with either anti-CYP2C9 or anti-CYP3A4 were incubated with purified CYP2C9, CYP3A4, or CYP2C9-CYP3A4 mixture for 8 h. Ten micrograms of each isoform, diluted in a total volume of 250 μ l of binding buffer, was added to the vials. The proteins were in 100 mM potassium phosphate buffer, 20% glycerol, pH 7.4. No detergents were present in the preparation. Unbound protein was washed away by repeatedly washing with binding buffer, followed by separation of the protein complex from the antibody using elution buffer. Because CYP2C9 and CYP3A4 are both of $\sim$ 57 kDa molecular mass, they would appear together on an SDS-polyacrylamide gel electrophoresis blot. Hence, the proteins were probed directly on a dot blot to identify the isoforms present (CYP2C9 alone, CYP3A4 alone, or CYP2C9-CYP3A4 complex). In brief, 5 μ l of each eluted protein sample was blotted onto a nitrocellulose membrane and allowed to air dry for 2 h. The membrane was then blocked with Tris-buffered saline + 5% milk for 1 h, blotted with anti-CYP3A4 (primary antibody/TBST ratio 1:1000) for 1 h, washed three times for 10 min with TBST, blotted with secondary antibody (goat anti-rabbit/TBST ratio 1:10,000) for 1 h, washed three times for 10 min with TBST, and then developed using 1:1 chemiluminescent reagents A and B, provided in the kit.

Results

Effect of CYP3A4 on CYP2C9 Activity. CYP3A4 strongly inhibited the activity of CYP2C9 when either (*S*)-flurbiprofen (Fig. 1) or (*S*)-naproxen (Fig. 2) was used as substrate. Figures 1A and 2A depict the kinetics of the respective reactions at 10 pmol of CPR/incubation, whereas Figs. 1B and 2B depict the reactions at 20 pmol of CPR/incubation. Flurbiprofen hydroxylation displayed hyperbolic kinetics, whereas naproxen demethylation was characterized by biphasic kinetics, indicative of two substrate binding sites. Up to 74 and 84% inhibition was observed with flurbiprofen and naproxen metabolism, respectively. Table 1 summarizes the average percentage inhibition observed from triplicate experiments. Doubling the amount of

FIG. 1. Effect of CYP3A4 on the activity of CYP2C9 using flurbiprofen as a substrate, using 10 and 20 pmol of CPR in A and B, respectively. Data fitting for the plots is shown. For clarity, representative data from a single experiment are presented. However, all the experiments were conducted in triplicate over three separate days, and the mean values were used for inhibition degree determination.

CYP3A4 from 5 to 10 pmol/incubation reduced the percentage of CYP2C9 residual activity from 37.5 to 26.2% and 29 to 16% for flurbiprofen and naproxen, respectively. When 20 pmol of CPR was used and CYP3A4 levels doubled, the residual activity reduced from 57.6 to 35.8% and 35 to 25% for flurbiprofen and naproxen, respectively. Increasing the amount of CPR in the incubations from 10 to 20 pmol/incubation only very modestly reduced the amount of inhibition observed, suggesting that the inhibition observed was not because of a lack of sufficient CPR present. In all the cases, only a minimal effect of CYP3A4 on the K_m of the reactions was noted (Figs. 1 and 2). Control incubations with flurbiprofen and only CYP3A4 indicated that CYP3A4 did not metabolize flurbiprofen.

rCPR was also evaluated as an alternative to hCPR in the CYP2C9-CYP3A4 interaction experiments, and essentially similar inhibition was noted (Table 1). For cost and availability reasons, rCPR was used in all the subsequent activity experiments involving flurbiprofen as a substrate. The effect of b5 on the CYP2C9-CYP3A4 interactions was examined because the presence of the redox protein b5 can increase P450 metabolism rates for certain substrates and enzymes, particularly CYP3A4. In addition, b5 can also bind to both proteins, hence potentially affecting the CYP2C9-CYP3A4. Figure 3 depicts CYP2C9-mediated metabolism of flurbiprofen in the presence of b5, CPR, and CYP3A4, and the percentage inhibition data from these

FIG. 2. Effect of CYP3A4 on the activity of CYP2C9 using naproxen as a substrate, using 10 and 20 pmol of CPR in A and B, respectively. Data fitting for the plots is shown. For clarity, representative data from a single experiment are presented. However, all the experiments were conducted in triplicate over three separate days, and the mean values were used for inhibition degree determination.

TABLE 1
Summary of CYP2C9 inhibition by CYP3A4

CYP2C9/CYP3A4/CPR/b5	Isoform Being Tested	Substrate	Percentage Inhibition in V_{max}
1:1:2:0	CYP2C9	Naproxen	71 ± 3.46
1:2:2:0	CYP2C9	Naproxen	84 ± 0
1:1:4:0	CYP2C9	Naproxen	65 ± 5.7
1:2:4:0	CYP2C9	Naproxen	75 ± 0
1:1:2:0	CYP2C9	Flurbiprofen	62.5 ± 4.6
1:1:2:0 rCPR	CYP2C9	Flurbiprofen	65.3 ± 2.1
1:2:2:0	CYP2C9	Flurbiprofen	73.8 ± 5.5
1:1:4:0	CYP2C9	Flurbiprofen	42.4 ± 5.8
1:1:4:0 rCPR	CYP2C9	Flurbiprofen	41.6 ± 6.2
1:2:4:0	CYP2C9	Flurbiprofen	64.2 ± 5.5
1:1:2:1	CYP2C9	Flurbiprofen	54.6 ± 2.6

experiments are presented in Table 1. It is noteworthy that b5 only marginally affected the amount of inhibition of CYP2C9 activity.

Effect of CYP2C9 on CYP3A4 Activity. Linearity of CYP3A4 metabolism of TST was observed for up to 10 min and up to 0.1 μ M protein (data not shown). Inclusion of CYP2C9 in the incubation mixtures resulted in only minimal inhibition of CYP3A4-mediated TST metabolism (Fig. 4), in contrast to the results observed with the effect of CYP3A4 on CYP2C9 activity. Inclusion of b5 had no additional effect. In control experiments using CYP2C9 only, a small

FIG. 3. Effect of b5 on CYP2C9/CYP3A4 interactions, using flurbiprofen as a substrate. Data fitting for the plots is shown. For clarity, representative data from a single experiment are presented. The open circles are data from experiments without CYP3A4 and with b5; the closed circles are from experiments with CYP3A4 and b5; and the open triangles are from experiments with CYP3A4 and without b5.

FIG. 4. Effect of CYP2C9 on the activity of CYP3A4 using TST as a substrate. Data fitting for the plots is shown. The data points are averages and S.D. of triplicates.

amount of hydroxylated TST metabolites was observed but was below quantification limits (0.2 pmol of metabolite/min/pmol CYP3A4).

Interactions between Truncated CYP2C9 and CYP3A4 Proteins. To assess whether the hydrophobic N terminus of these proteins might be involved in the observed interactions, CYP2C9-CYP3A4 interaction experiments were repeated with truncated versions of the protein that were lacking the membrane binding N-terminal domain. In contrast to results with full-length protein, when CYP2C9(t) and full-length CYP3A4 were incubated with flurbiprofen as a substrate, no inhibition was observed (Fig. 5, inset). This same lack of inhibition was also observed when CYP2C9 (full-length) and CYP3A4(t) enzymes were used (Fig. 5). These results establish that the hydrophobic N terminus of these two proteins is involved in the observed interactions.

Coimmunoprecipitation of CYP2C9-CYP3A4 Heteromers. To isolate a CYP2C9-CYP3A4 heteromer complex, coimmunoprecipitation experiments followed by a dot blot analysis were performed. Figure 6 depicts the dot blot analysis probed with anti-CYP3A4. Spots 1 and 2 represent the CYP2C9-CYP3A4 complex incubated with anti-CYP2C9 during the immunoprecipitation step; spots 3 and 4 contained only CYP2C9 or CYP3A4 incubated with anti-CYP2C9, respectively (i.e., negative controls); spots 5 and 6 contained the CYP2C9-CYP3A4 complex incubated with anti-CYP3A4 (positive controls); spot 7 contained CYP3A4 incubated with anti-CYP3A4 (positive control); spot 8 contained CYP2C9 incubated with anti-CYP3A4 (negative control); and spot 9 contained no antibody inclu-

FIG. 5. Effect of truncation on the CYP2C9-CYP3A4 interactions. The inset graph shows the interaction of CYP2C9(t) with CYP3A4, whereas the main graph shows the interaction of CYP2C9 with CYP3A4(t). Data fitting for the plots is shown. For clarity, representative data from a single experiment are presented. However, all the experiments were conducted in triplicate over three separate days.

FIG. 6. Dot blot of CYP2C9-CYP3A4 heteromer formation after coimmunoprecipitation of the proteins. The membrane was blotted with anti-CYP3A4. Spots 1 and 2 had CYP2C9-CYP3A4 complex incubated with anti-CYP2C9 during immunoprecipitation; spots 3 and 4 had only CYP2C9 and CYP3A4 incubated with anti-CYP2C9, respectively (negative controls); spots 5 and 6 had CYP2C9-CYP3A4 complex incubated with anti-CYP3A4 (positive controls); spot 7 had CYP3A4 incubated with anti-CYP3A4 (positive control); spot 8 had CYP2C9 incubated with anti-CYP3A4 (negative control); and spot 9 had no antibody during immunoprecipitation (negative control).

sion during immunoprecipitation (negative control). Hence, there are negative controls to ensure that anti-CYP2C9 does not recognize CYP3A4 and anti-CYP3A4 does not recognize CYP2C9 during the immunoprecipitation. In addition, a negative control showed that anti-CYP3A4 does not recognize CYP2C9 at the dot blot stage, and a final negative control ensures there is no nonspecific binding to the resin. These negative controls were designed to ensure that the dots seen in samples 1 and 2 are real and not nonspecific binding or artifacts. All three positive controls (spots 5–7) produced positive results, whereas all four negative controls (spots 3, 4, 8, and 9) exhibited very little evidence of protein. Because an equivalent signal was noted for spot 9 that contained no antibody in the immunoprecipitation step, it was interpreted that the minimal amount of “protein” observed in the negative control spots was most likely the result of nonspecific binding. Spots 1 and 2, which contained the CYP2C9-CYP3A4 mixture incubated with anti-CYP2C9 antibody and probed with anti-CYP3A4 antibody, gave a strong signal, providing evidence

FIG. 7. Dot blot of CYP2C9-CYP3A4(t) after coimmunoprecipitation of the proteins. The membrane was blotted with anti-CYP3A4. Spots 1 and 2 had CYP2C9 plus CYP3A4(t) incubated with anti-CYP2C9 during immunoprecipitation; spot 3 had CYP2C9 plus CYP3A4(t) incubated with anti-CYP3A4 (positive control); spots 4 and 5 had only CYP2C9 and CYP3A4 incubated with anti-CYP2C9, respectively (negative controls); spot 6 had CYP2C9 incubated with anti-CYP3A4 (negative control); spot 7 had CYP3A4 incubated with anti-CYP3A4 (positive control); and spot 8 had no antibody during immunoprecipitation (negative control).

that CYP2C9 and CYP3A4 associate with each other physically, resulting in the formation of heteromers. Coimmunoprecipitation with CYP2C9 and CYP3A4(t) showed the absence of any heteromer formation (Fig. 7).

Discussion

It has been previously shown that protein-protein interactions involving CYP2C9 and CYP2D6 resulted in a protein dose-dependent inhibition of CYP2C9 activity when CYP2D6 was coincubated (Subramanian et al., 2009). Additional results from these studies evaluating order of mixing of the proteins provided indirect evidence for the formation of CYP2C9-CYP2D6 heteromers. This interaction was unidirectional as CYP2D6 altered the activity of CYP2C9, but not vice versa. The present study builds on that work to show that CYP2C9 activity can also be modulated to an even greater extent by CYP3A4, a P450 protein that is present in the liver in much greater abundance than CYP2D6 (Shimada et al., 1994).

As depicted in Figs. 1 and 2 and summarized in Table 1, CYP3A4 strongly inhibits CYP2C9 activity by up to 84%. This inhibition of CYP2C9 activity occurred in a protein dose-dependent fashion for both CYP2C9 substrates tested (flurbiprofen and naproxen), although the degree of inhibition observed was somewhat substrate-dependent. The dissociation (K_d) for the interaction of CYP2C9 with CPR was reported to be 33 nM (Locuson et al., 2007), similar to the K_d value of 20 nM reported for the interaction of CYP3A4-CPR (Shimada et al., 2005). The similarity in K_d values seems to preclude competition for CPR as a sole factor governing CYP2C9-CYP3A4 interactions. In addition, experiments were conducted with up to 1:8 ratio of P450/CPR, and full activity was not restored (data not shown).

CYP2C9 did not affect the activity of CYP3A4, in agreement with previous work on the effects of human CYP2C9 on human CYP3A4 (Yamazaki et al., 1997). In other work involving human P450 enzymes, CYP2C9 inhibited methoxychlor-*O*-demethylation by CYP2C19, whereas CYP2C19 activated diclofenac hydroxylation by CYP2C9 (Hazai and Kupfer, 2005). These types of human P450-P450 interactions resulting in altered activity have also been

shown through effects of CYP1A1, full-length CYP1A2, and truncated CYP1A2 on CYP3A4, but CYP2C9, CYP2E1, and CYP2D6 failed to affect CYP3A4 activity (Yamazaki et al., 1997). Because expressed enzyme systems are sometimes used for making in vitro/in vivo correlations of intrinsic clearances, or mixtures of expressed enzymes may be used to evaluate drug-drug interactions, these findings may be of predictive importance because the absence of a complete complement of P450 isoforms in an expressed system may lead to erroneous predictions. To date, the clinical relevance of these protein-protein interactions in humans remains unclear. However, recent work in livers from adults with various stages of liver disease has shown that CYP2C9 protein expression did not change with liver disease but that CYP2C9 activity increased significantly (Fisher et al., 2009). It is tempting to speculate that this change in CYP2C9 activity in the absence of a change in CYP2C9 protein but increases and decreases in other P450 protein expression may have been the result of P450-P450 interactions.

Observation of direct interactions between two P450s by biophysical methods has been challenging given the lipophilic environment required to solubilize P450s and their inherent tendency to aggregate. Analytical ultracentrifugation studies with CYP2C9 and other proteins have shown that these P450 proteins exist in a highly heterogeneous environment, forming up to 20mers (M. Subramanian and T. S. Tracy, unpublished data; Guengerich and Holladay, 1979; French et al., 1980; Tsuprun et al., 1986; Myasoedova and Tsuprun, 1993; Black and Martin, 1994; Kempf et al., 1995; Davydov et al., 2005). Chemical cross-linking and bimolecular fluorescence complementation spectroscopy studies have shown the formation of CYP1A1-CYP3A2 and CYP2E1-CYP2C2 complex formation, respectively (Alston et al., 1991; Davydov et al., 2005; Ozalp et al., 2005). Direct evidence of physical interactions between CYP2C9 and CYP3A4 is provided by coimmunoprecipitation studies (Fig. 6), providing evidence of a CYP2C9-CYP3A4 heteromer complex and suggesting that these direct interactions could be responsible for the observed changes in activity. Although expressed P450s are known to aggregate in vitro, particularly at higher concentrations, the results from our coimmunoprecipitation experiments with truncated enzymes showing no interaction suggest that direct interactions do occur with the full-length proteins and the interactions are not purely nonspecific (Fig. 7). However, the coimmunoprecipitation experiments were performed in the absence of any lipids. Determining whether CYP2C9 and CYP3A4 form a heteromer within a lipid milieu would require further coimmunoprecipitation studies with either lipid micelles or microsomal proteins. In vivo, whether the N terminus is involved in protein-protein interactions or simply anchors the protein in membrane to allow for interactions at other hydrophobic regions remains unclear. It is possible that interactions at the N terminus may be necessary for other hydrophobic interactions to also occur.

To determine the specific domains of CYP2C9 and CYP3A4 that were interacting, incubations were conducted wherein one of the isoforms was truncated. The "full-length" CYP2C9 used contained modifications for expression in *E. coli*, wherein the first 7 codons were modified (Barnes et al., 1991), whereas the full-length CYP3A4 had residues 3 to 12 deleted (Gillam et al., 1993). The first 30 amino acids that code for the N-terminal membrane binding hydrophobic region were removed in the truncated versions of CYP2C9 and CYP3A4 used herein. As depicted in Fig. 5, all inhibition was abolished when either CYP2C9 or CYP3A4 was truncated, proving that these interactions require both N termini to be intact. CYP2C9 and CYP3A4(t) coimmunoprecipitation failed to show formation of a heteromer complex, further corroborating the hypothesis that both the N termini need to be intact for an interaction. In the absence of the N

terminus, the two proteins may be unable to directly interact through the N terminus, or because the truncated protein is unable to insert itself into the lipid milieu and hence is incapable of interacting. Previous studies have shown that CPR and b5, lacking their hydrophobic membrane binding N termini and C termini, respectively, were unable to provide electrons to P450s, indicating the relevance of nonspecific hydrophobic interactions in P450-CPR and P450-b5 interactions (Black et al., 1979; Lee-Robichaud et al., 1997; Chudaev et al., 2001; Mulrooney et al., 2004). Other studies of P450-P450 and P450-redox interactions studies have suggested the importance of electrostatic forces (Shimizu et al., 1991; Shen and Strobel, 1993; Omata et al., 1994; Bridges et al., 1998; Chudaev et al., 2001; Mulrooney et al., 2004; Kelley et al., 2005). Combining the information on P450-P450 interactions and P450-redox partner interactions, it could be speculated that hydrophobic forces within the cell membrane may initiate the interactions between two P450s, aligning the two enzymes in a spatial configuration favorable for electrostatic interactions to follow.

In summary, a CYP2C9-CYP3A4 heteromer complex has been identified, indicating that these two key isoforms can directly interact via their N termini, in a manner that reduces the activity of CYP2C9 by up to 84%. The mechanisms governing these interactions, their effect on in vitro/in vivo correlations, and physiological significance warrant further investigation.

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