Targeting of Splice Variants of Human Cytochrome P450 2C8 (CYP2C8) to Mitochondria and Their Role in Arachidonic Acid Metabolism and Respiratory Dysfunction*

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Abstract

In this study, we found that the full-length CYP2C8 (WT CYP2C8) and N-terminal truncated splice variant 3 (~44-kDa mass) are localized in mitochondria in addition to the endoplasmic reticulum. Analysis of human livers showed that the mitochondrial levels of these two forms varied markedly. Molecular modeling based on the x-ray crystal structure coordinates of CYP2D6 and CYP2C8 showed that despite lacking the N-terminal 102 residues variant 3 possessed nearly complete substrate binding and heme binding pockets. Stable expression of cDNAs in HepG2 cells showed that the WT protein is mostly targeted to the endoplasmic reticulum and at low levels to mitochondria, whereas variant 3 is primarily targeted to mitochondria and at low levels to the endoplasmic reticulum. Enzyme reconstitution experiments showed that both microsomal and mitochondrial WT CYP2C8 efficiently catalyzed paclitaxel 6-hydroxylation. However, mitochondrial variant 3 was unable to catalyze this reaction possibly because of its inability to stabilize the large 854-Da substrate. Conversely, mitochondrial variant 3 catalyzed the metabolism of arachidonic acid into 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids and 20-hydroxyeicosatetraenoic acid when reconstituted with adrenodoxin and adrenodoxin reductase. HepG2 cells stably expressing variant 3 generated higher levels of reactive oxygen species and showed a higher level of mitochondrial respiratory dysfunction. This study suggests that mitochondrially targeted variant 3 CYP2C8 may contribute to oxidative stress in various tissues.

Background: Human CYP2C8 is involved in the metabolism of >20% of drugs on the market.

Results: Both full-length (WT) CYP2C8 and splice variant 3 are bimodally targeted to mitochondria.

Conclusion: Mitochondrial CYP2C8 metabolizes paclitaxel and arachidonic acid (ω-hydroxylation).

Significance: Mitochondrial CYP2C8 is likely to play role in ischemic injury and oxidative stress.
of linoleic acid to products that can have cytotoxic vasoconstrictive effects (14–16). Indeed, transgenic overexpression of CYP2C8 in cardiomyocytes increased necrosis in a Langendorff in vitro heart perfusion system (17–19) and an associated increase in the production of the leukotxin diols 9,10- and 12,13-dihydroxyoctadecenoic acid and ROS. Furthermore, trimethoprim, a selective inhibitor of human CYP2C8, improved left ventricular developed pressure recovery and reduced infarct size after ischemia-reperfusion in isolated CYP2C8-overexpressing mouse heart preparations (20). Notably, infarct size was also reduced to control levels by the antioxidants $N$-acetylcysteine and superoxide dismutase, suggesting a role for ROS in CYP2C8-mediated susceptibility to ischemia-reperfusion injury. However, the precise mechanism of the cardiomyodulatory function of CYP2C8 remains unclear.

Several studies from our and other laboratories have demonstrated that various cytochrome P450 members of Family 1, 2, and 3 CYPs are associated with mitochondria in addition to the endoplasmic reticulum (ER) (21–23). Studies from our laboratory have described the mechanisms of bimodal targeting of CYP1A1, -1B1, -2B1, -2E1, and -2D6 to mitochondria and ER (24–32). The bimodal targeting of these proteins is driven by their N-terminal “chimeric signals” resulting in their dual targeting properties to both ER and mitochondria. CYPs localized in the mitochondrial compartment catalyze drug metabolism, efficiently interacting with mitochondrial adrenodoxin (Adx) and adrenodoxin reductase (AdxR) (25, 26, 28). In the case of CYP2B1, -2D6, and -2E1, activation of cryptic mitochondrial targeting signals by protein kinase A- or protein kinase C-mediated phosphorylation is required at sites immediately flanking the targeting signal and/or membrane-anchoring regions (26–30). However, activation by endoproteolytic cleavage by a cytosolic endoprotease is required in the cases of CYP1A1 and CYP1B1, exposing the mitochondrial signal (24, 31, 32). The truncated forms of CYP1A1 and CYP1B1 exhibit altered substrate specificity compared with the microsomal counterparts (24, 31, 32). However, it remains unclear whether CYP2C8 is also targeted to mitochondria and whether the mitochondrial form plays a role in drug metabolism and drug-induced toxicity.

Most studies on genetic polymorphic forms of CYP2C8 are focused on the mutations targeted to the catalytic domain of the enzyme (7, 11–13). First, analysis of human liver samples in this study showed that, similar to some other Family 1 and 2 CYPs, CYP2C8 can also be localized in the mitochondria. Second, a nearly full-length form (comparable in size with the microsomal CYP2C8; designated as Var_1) and a truncated form (44 kDa; designated as Var_3) were detected in mitochondria. Notably, despite missing the critical Arg-97 residue involved in binding to the heme group in the full-length enzyme (33), the N-terminal truncated form is capable of binding to heme and catalyzed the metabolism of some but not all CYP2C8 substrates. In this study, we show that three major splice variants of CYP2C8 expressed in human liver exhibit remarkable differences with respect to relative levels of microsomal (ER) and mitochondrial targeting, metabolism of paclitaxel and arachidonic acid, and induction of cellular oxidative stress.

**MATERIALS AND METHODS**

**Isolation of Mitochondria and Microsomes from Frozen Human Liver Samples**—Human liver samples were obtained through Tennessee Donor Services (Nashville, TN) and used in accordance with Vanderbilt Institutional Board guidelines. Mitochondria and microsomes were isolated from human liver samples by using a modification of a method described previously (29, 30). Briefly, livers were washed in ice-cold saline and homogenized in 10 volumes of sucrose-mannitol buffer (20 mM potassium HEPES buffer (pH 7.5) containing 70 mM sucrose, 220 mM mannitol, 2 mM EDTA, and 0.5 mg/ml BSA). Mitochondrial and microsomal fractions were isolated from the homogenates using a differential centrifugation method (24, 34). Mitochondria were pelleted at 8,000 $\times$ g for 15 min. Crude mitochondria were washed twice with the above buffer and pelleted through a 0.8 M sucrose layer at 14,000 $\times$ g for 30 min, and the mitochondrial pellet was washed twice with sucrose-mannitol buffer. Mitoplasts were prepared by treatment with digitonin (75 $\mu$g/mg protein; Calbiochem) at 4°C for 10 min. The resulting mitoplast pellet was washed twice with sucrose-mannitol buffer. Microsomes were isolated from the postmitochondrial supernatant by centrifugation at 100,000 $\times$ g for 60 min at 4°C. All subcellular preparations were resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride and frozen at $-80^\circ$ C.

**Characterization of Human CYP2C8 Variants**—Following the CYP nomenclature of Nelson et al. (35), the three different molecular forms characterized in this study are: WT CYP2C8 containing all nine exons (hereafter called Var_1), differentially spliced $\Delta$1ade2a (Var_2), and differentially spliced 1n$\Delta$1a$\Delta$2a (Var_3). The nucleotide sequences of the WT CYP2C8 (GenBank$^{TM}$ accession number NM_000770.3) and two reported transcripts Var_2 (GenBank accession number NM_001198853.1) and transcript Var_3 (GenBank accession number NM_001198854.1) were aligned by a Clustal format alignment, and common 5’ and 3’ internal primers were generated so that all three forms of CYP2C8 (Var_1, Var_2, and Var_3) were amplified in one single PCR. Total RNA was isolated from human liver samples using TRIzol reagent in accordance with the manufacturer’s instructions (Invitrogen). Total RNA (5 $\mu$g) was reverse transcribed using a High Capacity cDNA Archive kit (Applied Biosystems, Carlsbad, CA), and 100 ng was used for PCR amplification using the sense 5’-GTCCTGGTGCTGTG- and antisense 5’-GAAACGCCGGATC-TCCTCTTTTAT-3’ and antisense 5’-GAAAGCGCGGATCTCCTTCATC-3’ primers from the region conserved in all three mRNAs. The amplicons were resolved by electrophoresis on 1.5% agarose gels and cloned in a TOPO vector using a TOPO TA cloning kit (Invitrogen), and the nucleotide sequences of all three cDNA amplicons were confirmed by nucleotide sequence analysis.

**Molecular Modeling of CYP2C8**—The structure of human CYP2C8 Var_3 was modeled using the online protein structure prediction server Phyre 2 (36). The best model was aligned with the available CYP2C8 structure complexed with felodipine (Protein Data Bank code 2NNJ) using the PyMOL Molecular Graphics System (Version 1.5.0.4, Schrödinger). The putative
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heme-binding residues of WTCY2C8 and Var_3 were predicted based on the known structures of CYP2C5 and CYP2C8 (33, 37, 38).

Construction of WT and Variant CYP2C8 cDNAs—The ORF clone of human CYP2C8 (RG204605) was purchased from OriGene Technologies, Rockville, MD. Var_2 and Var_3 reading frames were amplified and cloned in the same pcMV6-AC-GFP vector (Precision Shuttle mammalian vector with C-terminal truncated GFP tag) in SgfII and MluI restriction sites. For stable cell expression, wild-type CYP2C8 (Var_1), Var_2, and Var_3 cDNAs were cloned in pGp-lenti viral plasmid (GenePass, Nashville, TN) in BamHI and AvrII restriction sites. An internal BamHI site was conservatively mutated using a QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). PCR amplification was carried out using a GeneAmp XL PCR kit (Applied Biosystems/Roche Applied Science) following the manufacturer's suggested protocol.

In Vitro Transport of 35S-Labeled Protein into Isolated Mitochondria—The three cDNA constructs (in pcMV6-AC-GFP vectors) were used as templates in T7 polymerase-coupled rabbit reticulocyte lysate transcription-translation systems (Promega, Madison, WI) in the presence of [35S]Met as described previously (24). Import of 35S-labeled translation products in rabbit reticulocyte lysate was carried out as described by Gasser et al. (39) and modified by Bhat and Avadhani (40) and Addya et al. (24) using freshly isolated rat liver mitochondria. For some control experiments, mitochondria were preincubated with carbonyl cyanide mchlorophenylhydrazone (50 μM; Sigma-Aldrich) or oligomycin (50 μM; Sigma-Aldrich) at 25 °C for 20 min prior to initiating the import reaction. After import, trypsin digestion of mitochondria was performed for 20 min on ice (150 μg of trypsin/mg of mitochondrial protein). Control mitochondria were incubated similarly without added trypsin. Soybean trypsin inhibitor (1.5 mg/mg of protein) was added to all samples to terminate the reactions. Mitochondria from both trypsin-treated and untreated samples were reisolated by pelleting through 0.8M sucrose, and the proteins were subjected to SDS-PAGE followed by fluorography.

Transient Transfection and Protease Protection Assay—Transfection of COS-7 cells with cDNA constructs (6 μg/100-mm² plate) was carried out using the lipophilic reagent FuGENE 6 (Roche Applied Sciences). Cells were harvested 48 h post-transfection and used for isolating subcellular fractionations. Mitochondria and microsomes were suspended at 10 mg/ml in 50 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol and subjected to trypsin digestion (75 μg/mg of protein) for 20 min on ice. The reaction was stopped by adding a 10-fold excess of trypsin inhibitor and an equal volume of 2× Laemmli sample buffer (41). The samples were incubated at 95 °C for 5 min and subjected to SDS-PAGE (12%, w/v) for immunoblot analysis.

Immunofluorescence Microscopy—Cells were fixed with methanol, permeabilized by treatment with 0.1% Triton X-100 (v/v), blocked with 5% goat serum (v/v), and stained with appropriate primary and secondary antibodies. Fluorescence microcopy was done using an Olympus BX61 microscope, and Pearson’s coefficient was calculated using Velocity 5.3 software.

Reconstitution of Purified Human CYP2C8 for Paclitaxel Hydroxylation—Human CYP2C8 expressed in Escherichia coli cells was purified as described before (42, 43), and the CYP content was measured by the Fe⁺⁺-CO difference spectra described later in this section. Enzyme reactions with purified CYP2C8 (full-length form) were performed in 0.3-ml final volumes of 50 mM potassium phosphate (pH 7.4) buffer containing 0.2 nmol of purified CYP protein reconstituted with or without 0.5 nmol of cytochrome P450 reductase or 0.4 nmol of purified Adx and 0.04 nmol of purified AdXR and 10 μM paclitaxel. Reconstitutions with cytochrome P450 reductase also contained phospholipid vesicles (42, 43). Inhibition studies were carried out by preincubating the enzyme for 20 min on ice with 10 mg/ml inhibitory antibody (BD Gentest, BD Biosciences) or similar amounts of control mouse ascites fluid or 5 μM montelukast at 37 °C for 20 min. Reactions were initiated by addition of 1 mM NADPH and carried out for 20 min at 37 °C in a shaking water bath. Reactions were terminated by addition of 150 μl of 100% CH₃CN, mixed well using a vortex device, and centrifuged at 9,000 × g for 20 min at 4 °C. Approximately 250–300 μl of clear supernatant was transferred to a fresh tube and frozen at −80 °C until analysis.

Paclitaxel Oxidation by Mitochondrial and Microsomal Enzymes from Stable Cell Lines—Reactions were run in 0.3-ml final volumes of 50 mM potassium phosphate (pH 7.4) buffer using 300–500 μg of freeze-thawed mitochondrial protein or microsomal protein. Mitochondrial samples were reconstituted with 0.4 nmol of purified Adx and 0.04 nmol of purified AdxR for supplementing the loss of Adx and AdxR during mitochondrial isolation (44). Microsomal samples were not supplemented with cytochrome P450 reductase because they contain optimal levels of NADPH-cytochrome P450 reductase for reconstitution of enzyme activity. Either 10 μM paclitaxel or 70 μM arachidonic acid was used as a substrate. Inhibition studies were performed by preincubating enzymes for 20 min with 1.5 mg of inhibitory antibody for 20 min on ice or 5 μM montelukast at 37 °C for 20 min. Reactions were initiated by addition of 1 mM NADPH and continued for 20 min at 37 °C for paclitaxel and 5 min at 37 °C for arachidonic acid in a shaking water bath. Reactions with paclitaxel were processed as described above, and those with arachidonic acid were snap frozen at −80 °C until analysis.

LC-MS Analysis of Arachidonic Acid Products—To each 1.0-ml aliquot of cell culture, 1.0 ml of 0.15 M KCl was added along with 15 μl of 0.5 mM triphenylphosphine (in CHCl₃; CH₃OH, 2:1, v/v), and 9 μg of 20-[2H₂]-hydroxyeicosatetraenoic acid (HETE), and 9 μg each of 8,9-[2H₃]EET, 11,12-[2H₃]EET, and 14,15-[3H₂]EET. To this, 4.0 ml of a mixture of CHCl₃:CH₃OH (2:1, v/v) and 116 μl of HCl were added. After mixing with a vortex device, the organic layer was transferred to a new tube, and the aqueous layer was extracted again with 5 ml of CHCl₃. The combined organic layers were pooled, 100 μl of 5 M KOH was added, and the samples were evaporated to dryness under a stream of N₂ at 45 °C. Each sample was dissolved in 1.0 ml of 0.4 M KOH in CH₂OH·H₂O (4:1, v/v), and the tubes were purged with argon gas and sealed (Teflon® liners). The samples were
heated at 50 °C for 45 min, and then 3.0 ml of 0.15 M KCl and 90 μl of glacial CH₃CO₂H were added to each. Each sample was extracted twice with diethyl ether, and the solvent was evaporated under a stream of N₂ at 45 °C. Each sample was dissolved in 2.0 ml of 0.5% CH₄CO₂H in hexane and loaded onto a silica cartridge pre-equilibrated with the same solvent followed by a 10-ml wash with the same solvent. The columns were eluted with 10 ml of a mixture of hexane:diethyl ether:CH₃CO₂H (50:50:0.5, v/v/v). The samples were dried under a stream of N₂ at 45 °C, 150 μl of 9 M aqueous CH₄CO₂H was added to each, and the samples were purged with argon gas and left at 23 °C overnight to convert the EETs to dihydrodiols. The samples were dried under a stream of N₂ and dissolved in 35 μl of a mixture of 15 mM aqueous NH₄CH₃CO₂ (pH 8.5):CH₃CN (1:1, v/v).

Aliquots were analyzed by LC-MS using a Waters Acquity octadecylsilane LC column (1.7 μm, 1.0 × 100 mm) operating at 60 °C. Solvent A was 15 mM aqueous NH₄CH₃CO₂ (pH 8.5), and Solvent B was CH₃CN. The following elution schedule was used: 0–1.0 min, 30% B (v/v), 0.18 ml/min flow; 1.0–5.0 min, 30–60% B, 0.18 ml/min flow; 5.0–5.2 min, 60–100% B, 0.20 ml/min flow; 5.2–6.0 min, hold at 100% B, 0.20 ml/min flow; 6.0–6.2 min, 100–30% B, 0.18 ml/min flow; and 6.2–8.0 min, 0.18 ml/min flow. The following ions were monitored in the MS² mode, all using a collision energy setting of 22: m/z 289 from m/z 319 for 20-HETE and from m/z 321 for 20-[2H₂]HETE, m/z 207 from m/z 337 for 14,15-dihydroxyeicosatrienoic acid and from m/z 340 for 14,15-[2H₃]dihydroxyeicosatrienoic acid, m/z 167 from m/z 337 for 11,12-dihydroxyeicosatrienoic acid and from m/z 340 for 11,12-[2H₂]dihydroxyicosanoic acid, and m/z 127 from m/z 337 for 11,12-dihydroxyeicosatrienoic acid and from m/z 340 for 11,12-[2H₃]dihydroxyicosanoic acid.

LC-MS Analysis of Paclitaxel Products—LC-MS analysis of the oxidation products was performed on a Waters Acquity UPLC system connected to a Thermo LTQ mass spectrometer (Thermo Fisher, Waltham, MA) using an Acquity UPLC BEH octadecylsilane (C₁₈) column (1.7 μm, 2.1 × 100 mm). LC conditions were as follows: Solvent A contained 0.1% HCO₂H acid in H₂O (v/v), and Solvent B contained 0.1% HCO₂H in CH₃CN (v/v). The following gradient program was used with a flow rate of 0.2 ml/min: 0–2 min, hold at 10% B (v/v); 2–7 min, linear gradient from 10% B to 90% B (v/v); 7–7.5 min, hold at 90% B (v/v); 7.5–8 min, linear gradient from 90% B to 10% B (v/v); 8–10 min, hold at 10% B (v/v). The temperature of the column was maintained at 40 °C. Samples (20 μl) were infused using an autosampler. MS analyses were performed in the electrospray ionization positive ion mode. The mass spectrometer was tuned using 6-hydroxyacridine. Product ion spectra were acquired over the range m/z 500–1000 and quantified using the target parent m/z 870, calibrating against external standards.

Generation of Stable Cell Lines—HepG2 cells were stably transduced with lentiviral vectors carrying Var_1 and Var_3 cDNAs. The lentiviral plasmids (12 μg each) and the packaging plasmid DNA (6 μg of Gag-Pol and MD.2G) were co-transduced into 293T cells using FuGENE HD transfection reagent to obtain fully functional lentiviral particles. After 48 h of transfection, the virus particles secreted in the cell culture medium were harvested by centrifugation at 600 × g for 5 min, filtered through a 0.2-μm syringe filter, and used for transducing HepG2 cells. The colonies were screened using puromycin (4 μg/ml) as the selection marker. Cell colonies were selected and amplified. All the experiments were conducted in cells that were cultured without puromycin for at least three passages to rule out the adverse effects of puromycin on mitochondrial function.

Subcellular Fractionation and Immunoblot Analysis—For the preparation of whole cell lysates, cells were lysed with radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl buffer (pH 7.4) containing 1% Nonidet P-40 (v/v), 0.25% sodium deoxycholate (w/v), 150 mM NaCl, and 1 mM EDTA plus protease inhibitors). The cell lysates (50 μg of protein) were used for immunoblotting to evaluate the level of protein after transient transfection. Mitochondria and microsomes from transfected cells were prepared essentially as described previously (24, 26, 44); suspended in 50 mM phosphate buffer (pH 7.4) containing 50 mM KCl, 10% glycerol (v/v), 0.1 mM dithiothreitol (DTT), and 0.1 mM PMSF; and stored at −80 °C. Proteins were solubilized in Laemmli sample buffer (39) containing 5% 2-mercaptoethanol (v/v) by incubation at 95 °C for 5 min, resolved by electrophoresis on SDS-polyacrylamide (10%, w/v) gels, and subjected to immunoblot analysis using 1:1,500 dilutions of primary antibody and 1:50,000 dilutions of IRDye-conjugated secondary antibodies. Blots were developed using an Odyssey scanner (LI-COR Biosciences, Bad Homburg, Germany) and quantified using NIH ImageJ software.

Alkaline Extraction of Membrane Proteins—The membrane topology of the mitochondrial and microsomal CYP2C8 was studied by extraction with alkaline Na₂CO₃ buffer essentially as described by Clark and Waterman (45) and modified by Anandatheerthavarada and co-workers (26, 28). Freshly isolated mitochondria and microsomes suspended in sucrose-mannitol buffer (10 mg of protein/ml) were diluted to 20-fold with 0.1 M Na₂CO₃ (pH 11) to a final concentration of 0.5 mg of protein/ml. This mixture was mixed with a Vortex device three times (30 s each) to obtain an even suspension. The suspension was incubated on ice for 30 min and then centrifuged at 210,000 × g for 90 min at 4 °C. The pellet was suspended in 10 mM Tris-HCl buffer (pH 7.0) containing 2% SDS (w/v), and dissociated proteins were subjected to immunoblot analysis. The supernatant was mixed with an equal volume of ice-cold 20% trichloroacetic acid (w/v) and held at 4 °C for 2 h. The precipitated proteins were collected by centrifugation at 100,000 × g for 20 min and washed twice with ice-cold acetone. The resulting protein pellet was dissolved in SDS-containing buffer. The pellet and soluble protein fractions (100 μg of protein) were subjected to immunoblot analysis.

Analysis of CO Difference Spectra—The CYP content of stable cell mitochondria and microsomes was measured by the Fe²⁺-CO versus Fe²⁺ difference spectra as described by Omura and Sato (46) and modified by Guengerich (47) using a dual beam spectrophotometer (Cary 1E; Varian, Walnut Creek, CA). Mitochondrial or microsomal proteins (0.3–0.5 mg) were solubilized in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mm EDTA, 20% glycerol (v/v), sodium cholate (0.5%, w/v), and Triton N-101 (0.4%, w/v).
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Quantitative Real Time PCR—For mRNA quantitation by PCR analysis, RNA was digested with Turbo DNase I (Ambion, Austin, TX). Total RNA (5 μg) was reverse transcribed using a High Capacity cDNA Archive kit, and 25 ng of the resulting cDNA was used in a standard Power SYBR Green real time PCR on an ABI 7300 real time PCR machine and analyzed using Primer Express 3.0 (Applied Biosystems). The levels of the integrated puromycin resistance gene (in relation to the cellular primer) were calculated using Student’s t test. A value of 0.05 was considered significant.

RESULTS

Localization of Full-length and Truncated CYP2C8 in Mitochondria and Microsomes of Human Liver Samples—We analyzed mitochondria and microsomes from 26 human liver samples for CYP2C8 levels by immunoblot analysis. Representative blots for 12 samples in Fig. 1A show that in addition to full-length CYP2C8 (apparent molecular mass of 54 kDa) there is a faster migrating (~44-kDa) species in many samples. Some samples also showed additional antibody reactive species of low abundance that may be additional molecular forms or degradation products. The relative levels of both 54- and 44-kDa species in the mitochondrial and microsomal fractions also markedly vary, suggesting interindividual variations. For example, samples HL97 and HL3718 showed very low mitochondrial 54-kDa protein but high microsomal content. Samples HL9016, HL3904, HL3987, and HL3053 showed relatively high mitochondrial 54-kDa protein and nearly equal microsomal levels of protein (Fig. 1A and quantification in B). The blots were co-developed with NADPH-cytochrome P450 reductase and TOM20 antibodies to evaluate the relative extent of cross-contamination of subcellular fractions used for analysis. The mitochondrial isolates from almost all human liver samples contain CYP2C8, ranging from 20 to 50% of the total tissue pool.

Immunoblots in Fig. 1A also show that the level of 44-kDa protein in the mitochondrial fraction was significant in many liver samples, whereas the level in the microsomal fraction was negligible in almost all samples. The ratios of mitochondrial 44-kDa species:54-kDa species ranged from 0.1 to 0.8 in different samples, indicating marked variability.

Alternatively Spliced mRNAs as a Source of Different CYP2C8 Molecular Species—At least three differentially spliced mRNAs for human CYP2C8 with open reading frames of 490, 420, and 388 amino acids have been reported (Ensembl Transcript ENST00000535898 and GenBank accession number NM_001198853.1). We therefore reasoned that some of the faster migrating proteins on SDS gels (Fig. 1A) could be translated from alternatively spliced mRNAs. The two predicted mRNAs (Var_2 and Var_3) lack different stretches of nucleotide sequences from exons I and II and are therefore predicted to yield three different sized amplicons on PCR amplification using both forward and reverse primers from conserved regions as indicated under "Materials and Methods." The splice junctions of the full-length and Var_3 mRNAs are shown in Fig. 2B. Both Var_2 and Var_3 mRNAs encode N-terminal truncated proteins compared with the full-length 54-kDa (Var_1) protein (Fig. 2, A and B). Var_2 and Var_3 showed predicted reading frames of 420 and 388 amino acids, respectively, compared with the 490-amino acid reading frame for full-length Var_1 protein. It should be noted that 11 transcripts, including additional splice variants not chosen for analysis, are derived by differential splicing of the CYPC8 gene-encoded primary transcript; eight of these are likely non-functional, three of them undergo nonsense-mediated decay, and the remaining five contain part of the introns and encode no protein products.

The agarose gel patterns in Fig. 2C show the relative levels of full-length Var_1, Var_2, and Var_3 mRNAs. Two important points are noteworthy regarding the Var_2 amplicon. First, it is
about 100 nucleotides larger than the full-length mRNA ampli-
con because within the mRNA region being amplified full-
length mRNA lacks a stretch of sequence that is present in
Var_2 mRNA. Second, the relative levels of Var_2 mRNA were
generally very low, consistent with the negligible to very low
steady-state levels of corresponding protein. For these reasons,
we did not pursue further characterization of this protein. The
Var_3 mRNA amplicon was seen in most liver samples and
represented a significant part of the total CYP2C8 mRNA pool
(Fig. 2C, panels i and ii). The relative distribution of Var_3 and
Var_1 amplicons showed marked variations in steady-state levels
as indicated by the ratios of band intensities (values in paren-
theses below the gel patterns). Samples 9016, B019, HL97, HL3718, and
9438 showed equal or marginally higher ratios of Var_3:Var_1.
Samples 9304, 500A, and 7017D showed higher levels of Var_1 and
lower levels of Var_3. Other samples, such as 3987 and HL111
showed low levels of both Var_1 and Var_3 amplicons. The low
levels of amplification with H111 RNA were surprising in view of
the high levels of both 54- and 44-kDa proteins detected in this
sample (Fig. 1A), although degraded RNA in the tissue may be the
reason. These results show marked variations in the levels of dif-
fferentially spliced Var_3 mRNA.

The possible molecular identity of Var_3 with the 44-kDa
protein detected in the human liver samples was verified by
immunoblot analysis of liver mitochondrial samples run along-
side stable HepG2 cells expressing Var_3 protein. Two liver
mitochondrial samples from each of the three panels in Fig. 1A
were used for electrophoresis. The immunoblot in Fig. 2D
shows that the 44-kDa protein co-migrates with the Var_3 protein
expressed in HepG2 cells. Notably, both the liver and
HepG2 cell mitochondria also show slower migrating nonspe-
cific protein bands.

Three-dimensional Molecular Modeling and in Silico Analysis of
Heme Binding Residues of Var_1 and Var_3 CYP2C8
Proteins—The next question we wanted to address was whether
Var_3 protein has an intact heme binding pocket. Using the

FIGURE 1. Different molecular forms of CYP2C8 in human liver samples. A, panels i–iii, representative immunoblots of mitochondrial and microosomal
proteins (50 μg each) from human liver samples (Mt, mitochondrial fraction; Mc, microsomal fraction; HL, human liver used in labeling of some of the samples).
Blots were developed with polyclonal antibodies to CYP2C8 (1:500 dilution, v/v) and TOM20 (1:2,000 dilution, v/v) and monoclonal antibody to cytochrome
P450 reductase (CPR) (1:1,500 dilution, v/v). In addition to the full-length CYP2C8, a smaller form of 44 kDa (Var_3 (V3)) was seen predominantly in the
mitochondrial fraction. The numbers in parentheses below the CYP2C8 immunoblot represent the ratios of Var_3 and full-length (Var_1) proteins in terms of
band intensities. B, relative distribution of full-length CYP2C8 in mitochondria and microsomes of the liver samples analyzed in A. The percent distribution was
calculated based on the densitometry of band intensities in A. Results represent averages from two blots.
CYP2C8 and CYP2D6 as the template (33, 36, 51), we generated the three-dimensional model for CYP2C8 (WT2C8; full length) and Var_3 (V3-2C8) and superimposed the two images (Fig. 3A, third panel). As seen from the superimposed image, the binding pocket and active site appear to be conserved in Var_3. However, because the F-G helices are missing, the substrate entrance tunnel is altered, which might alter entry and subsequently specificity toward different substrates.

Kelley and Sternberg (36) and Klein et al. (37) did a comprehensive analysis of more than 5,500 P450 heme-thiolate protein sequences of various species (PROSITE identifier PS00086; documentation PDOC00081) by calculating their consensus...
sequence. The authors conclude that family-specific signatures of the heme-binding region are more predictive as compared with the overall PROSITE pattern. For example, in CYP2D6, Arg-441 appears to be essential for its enzymatic function and heme binding. In consideration of this, because CYP2C also belongs to CYP2 family, we did a cluster sequence alignment of full-length CYP2D6, full-length (Var_1) CYP2C8, and CYP2C8 splice Var_3 to find the amino acids interacting with heme, and the results are summarized in Fig. 3C. Hydrogen-bonding interactions between the carboxyl groups of the two heme propionate groups and the side chains of Arg-97, Trp-120, Arg-124, His-368, Ser-429, and Arg-443 in Var_1 CYP2C8 (full length) and Trp-18, Arg-22, His-266, Ser-327, and Arg-341 of Var_3 CYP2C8 anchor the heme in the heme-binding site (Fig. 3B). The homology modeling of these proteins shows that the heme-binding Arg is well conserved in CYP2C8 with Arg at position 443 in wild type and position 341 in Var_3. Furthermore, all of the CYP proteins in CYTOCHROME_P450, PS00086 have a consensus pattern: \((F/W)\normal{(S/G/N/H)}\normal{X(G/D)}\normal{(F)}\normal{(R/K/H/P/T)}\normal{(P)}\normal{C(L/I/V/M/F/A/P)}\normal{(G/A/D)}\) where \(C\) is the heme iron ligand. This signature pattern is conserved in both WT CYP2C8 (428 – 437; FsGKRICAG) and splice Var_3 (326 – 335; FsGKRICAG). These results suggested that despite the absence of the N-terminal Arg-97 residue the Var_3 protein has the capability to bind the heme group. However, it is apparent that the lack of the F-G helical region creates a very wide substrate binding pocket, raising questions about its ability to bind large substrates.

**Mitochondrial Targeting of Full-length and Variant CYP2C8 Proteins Under in Vitro and in Cell Conditions**—The mitochondrial targeting efficiency of the WT, Var_2, and Var_3 proteins was studied with \textit{in vitro} import by isolated rat liver mitochondria as described previously (24, 40). The fluorogram presented in Fig. 4A, panel i, shows that all three molecular forms were imported into a trypsin-resistant compartment with Var_2 imported at the lowest efficiency (7% of input) and Var_3 at
the highest efficiency (~34% of input). In all three cases, the trypsin-resistant, putative imported molecular species co-migrated with the input protein on SDS gels, suggesting that the imported protein is not processed by mitochondrial matrix proteases. A control experiment showed that dihydrofolate reductase, a cytoplasmic protein, is not imported significantly, but SU9-dihydrofolate reductase protein (with an N-terminally fused mitochondrial targeting signal from ATPase subunit 9 (S2)) was imported efficiently (Fig. 4A, panel ii). As expected, the N-terminal cleavable SU9 targeting signal was clipped as part of the import process.

Subcellular targeting of the three proteins was studied by three different approaches. In the first approach, we used the MitoProt II-v.1.101 program to predict signal efficiencies. The Var_3 protein showed a mitochondrial export probability of 0.86, whereas the full-length Var_1 protein yielded a score of 0.39, and the variant 2 gave a score of 0.013 (Table 1). In the second approach, mitochondrial targeting was studied by transient transfection of COS-7 cells with the WT and variant cDNAs cloned in pCMV6-AC-GFP vector followed by subcellular fractionation. The immunoblot in Fig. 4B, panel i, shows the relative levels of expression of three cDNAs in COS-7 cells. Var_3 was expressed at a significantly higher level than the WT and Var_2 cDNAs. The mitochondrial (Mt) and microsomal (Mc) distributions of Var_1, Var_2, and Var_3 proteins in transfected cells are shown in Fig. 4B, panel ii. Both Var_1 and Var_2 proteins were targeted at higher levels to the microsomes than to mitochondria, whereas Var_3 protein was targeted at a substantially higher level to mitochondria. All three mitochondrial-associated proteins were relatively resistant to trypsin but became sensitive to trypsin following disruption of the mitochondrial membrane by treatment with Triton X-100 (Fig. 4B, panel iii, TT), suggesting that they are localized in the mitochondrial matrix compartment. Nearly 60–80% of mitochondrial-associated Var_1 and Var_3 proteins were resistant to trypsin, whereas only 5–10% of Var_2 protein was resistant to trypsin. A noteworthy point is that Var_3 protein appears as a doublet (Fig. 4B, panels i, ii, and iii) on gels. Although the precise reason remains unclear, it is likely due either to translation starting from an alternate position or to posttranslational modifications.

In the third approach, the level of mitochondrial targeting in COS-7 cells was further ascertained by confocal immunofluorescence microscopy of transfected cells. Transfected cells were stained with CYP2C8 antibody and co-stained with either an antibody to a mitochondrial marker (Fig. 4C, panel i, cytochrome-c oxidase subunit I (CcOII) or a microsomal marker (Fig. 4C, panel ii, calreticulin (CRT)), and the CYP staining patterns were superimposed on the cytochrome-c oxidase subunit I- or calreticulin-stained patterns. WT CYP2C8 co-localized with the mitochondrial marker with a marginal Pearson coefficient of 0.61. Var_2 protein co-localized insignificantly with the mitochondrial marker (Pearson coefficient of 0.4), whereas Var_3 protein co-localized with the highest Pearson coefficient of 0.9. Co-localization with the microsomal marker (calreticulin) was in the reverse order with WT and variant 2 exhibiting high efficiencies (Pearson coefficients of 0.82 and 0.9, respectively) and Var_3 protein exhibiting a very low efficiency (Pearson coefficient of 0.55). These results together suggest that although all three proteins localize to mitochondria and that there is a marked difference in relative efficiency with Var_3 showing the highest level, WT protein showing an intermediate level, and Var_2 protein showing the lowest level. This is consistent with our previous predictions on bimodal targeting of CYP1A1, -2E1, and -2D6 proteins to mitochondria that proteins with more hydrophilic N-terminal targeting domains with lower affinity of binding to the signal recognition particle are targeted to mitochondria at higher levels (28–32).

Reconstitution of Purified Full-length CYP2C8 with Microsomal and Mitochondrial Electron Transfer Proteins—The enzyme activity of purified CYP2C8 (Var_1) could be reconstituted with the mitochondrial electron transfer proteins Adx and AdxR using a large substrate, paclitaxel (854 Da), and a smaller substrate, dibenzylfluorescein (189 Da). Paclitaxel

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**TABLE 1**

| CYP2C8 | Protein sequence length | Probability of export to mitochondria |
|--------|------------------------|-------------------------------|
| CYP2C8 WT | 490 | 0.39 |
| CYP2C8 V*2 | 420 | 0.014 |
| CYP2C8 V*3 | 388 | 0.87 |

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**FIGURE 4.** Different subcellular targeting efficiencies of the three variant proteins. A, in vitro import of 35S-labeled translation products in isolated rat liver mitochondria. Panel i, 35S-labeled translation products of wild type (WT2C8), Var_2 (V*2-2C8), and Var_3 (V*3-2C8). Radiometric imaging of gels was performed to determine the level of import of input protein for each construct in trypsin-treated samples (T). The input protein level was considered to be 100% in each case. Panel ii, import of dihydrofolate reductase (DHFR) and SU9-dihydrofolate reductase (Su9-DHFR) proteins as negative and positive controls, respectively. The lanes marked “In” or “T” (for input) were loaded with 20% of the counts used for the import reactions. “C” represents control experiments in which total protein bound and imported into mitochondria is present. “T” represents trypsin-treated mitochondria in which only the protein imported into mitochondria is present. B, translocation of WT and variant 2 and 3 proteins in transiently transfected COS-7 cells. Panel i, total cell lysates (50 µg each) from transiently transfected COS-7 cells were resolved by 12% SDS (w/v)-PAGE and probed with antibodies to CYP2C8 and β-actin for assessing loading levels. Panel ii, mitochondria and microsomes were isolated from transfected COS-7 cells, and 50 µg of protein each was resolved by 12% SDS (w/v)-PAGE and probed with antibodies to CYP2C8, cytochrome P450 reductase (CPR), and cytochrome-c oxidase subunit I. Panel iii, relative resistance of mitochondrial-associated proteins to trypsin treatment (T). In some cases, mitochondria were lysed by treatment with 1% Triton X-100 (w/v) before trypsin treatment (T). Proteins (50 µg each) were resolved by SDS-PAGE and probed with antibodies to CYP2C8 and TOM20 for immunoblot analysis. C, immunofluorescence microscopy of COS-7 cells transfected with WT (Var_1), Var_2, or Var_3 cDNA. Panel i, a–c, co-localization of CYP2C8 with a mitochondrial marker, cytochrome-c oxidase subunit I (CcOII). Cells were stained with a 1:10,000 dilution (v/v) of primary anti-goat antibody to CYP2C8 (green) (Abcam, Cambridge, MA) and co-stained with a 1:500 dilution (v/v) of cytochrome-c oxidase subunit I (red)-anti-mouse antibody (Abcam). Panel ii, a–c, co-localization of CYP2C8 with microsomal membrane marker calreticulin (CRT) (anti-rabbit antibody) (red) (Santa Cruz Biotechnology, Santa Cruz, CA). The cells were subsequently incubated with secondary Alexa Fluor 546-conjugated anti-mouse and then anti-rabbit IgG and Alexa Fluor 488-conjugated anti-goat IgG and imaged through a confocal microscope. Numbers in the bottom panels indicate Pearson coefficients for coincidence calculated using Volocity 5.3 software.
Characterization of HepG2 Cell Lines Stably Expressing Human Full-length and Var_3 CYP2C8 Proteins—HepG2 cell lines stably expressing full-length (Var_1) and Var_3 cDNAs were generated using a lentiviral vector. Immunoblots (Fig. 6A) show the mitochondrial and microsomal protein levels in mock-transfected, WT CYP2C8, and CYP2C8 Var_3 cells. The microsomal protein content in Var_1 (WT) CYP2C8 cells was significantly higher than the mitochondrial content. In cells expressing CYP2C8 Var_3, however, the mitochondrial content was more than twice the microsomal level. Notably, in contrast to the overall level of expression of the two cDNA constructs in transiently transfected cells (Fig. 3), the steady-state level of Var_3 protein in the stable cell line was significantly lower. The reason for this difference remains unclear. The membrane topology of proteins associated with the mitochondrial fraction was evaluated using the alkaline Na₂CO₃ extraction method (26, 49). Both full-length and CYP2C8 Var_3 in the mitochondrial fraction were nearly quantitatively extracted with the alkaline aqueous phase, whereas the microsomal WT CYP2C8 protein partitioned more than into the insoluble fraction, suggesting a transmembrane topology. A small part of the microsomal Var_3 protein partitioned in the insoluble phase, suggesting that the bulk of the ER-associated Var_3 protein was associated mainly through membrane-extrinsic interaction. The PCR results in Fig. 6C show that all three cell lines contained nearly the same levels of integrated puromycin acetyltransferase gene, suggesting comparable levels of viral DNA integration.

The mitochondrial and microsomal heme contents of the stable cell lines were measured by ferrous CO difference spectra (Fig. 6, D and E). The WT CYP2C8 cell line showed a higher CYP content (~900 pmol/mg of protein) than the mitochondrial fraction (~700 pmol/mg of protein). The Var_3-expressing cells showed about 500 pmol/mg of protein, whereas the microsomal fraction from these cells showed <300 pmol/mg of protein. The mock-transfected cells showed very low CYP contents. The overall CYP contents of cell fractions from these cells are consistent with the relative CYP2C8 antibody-reactive proteins detected in these fractions (Fig. 6A). Both the mitochondrial and microsomal fractions from WT CYP2C8 cells showed comparable activity for paclitaxel 6-hydroxylation, which was inhibited by the CYP2C8-specific inhibitor montelukast and an inhibitory antibody (Fig. 6F). However, despite containing measurable CYP heme, mitochondria from CYP2C8 Var_3-expressing cells did not show any detectable paclitaxel 6-hydroxylation activity. We therefore decided to test the effects of the CYP2C8 inhibitors proadifen and montelukast on mitochondrial ROS production using a 2′,7′-dichlorodihydrofluorescein diacetate method. Both inhibitors and N-acetylcysteine, an antioxidant, marginally inhibited ROS production in isolated mitochondria from mock-transfected cells but significantly inhibited ROS production in mitochondria from WT CYP2C8- and CYP2C8 Var_3-expressing cells. The overall ROS production was substantially higher in the CYP2C8 Var_3-expressing cells. These results suggested that mitochondrial CYP2C8

6-hydroxylation activity was relatively higher with Adx plus AdxR electron transfer proteins, whereas dibenzylfluorescein oxidation was lower than the activity reconstituted with cytochrome P450 reductase (CPR). 0.4 nmol of purified Adx, 0.04 nmol of purified AdxR, and 10 μM paclitaxel in a 0.3-ml final volume as described under “Materials and Methods.” Montelukast (Mon) (5 μM) and inhibitory antibody to CYP2C8 (2C8Ab) (10 mg/ml) were used. Control ascites fluid (CAF; 10 mg/ml) was used as a negative control. B, reconstitution of dibenzylfluorescein oxidation was carried out essentially as described above in A. The activities in all cases represent the means ± S.E. (error bars) of three to five separate assays. Purified CYP2C8 was preincubated with inhibitors and control ascites fluid as described under “Materials and Methods.” * in A indicates no detectable activity.
Var_3 may be catalytically active and may accept electrons from Adx/AdxR.

We next tested the metabolic activity of WT CYP2C8 and CYP2C8 Var_3 with a smaller substrate, arachidonic acid, which belongs to a group of polyunsaturated fatty acids that are present in the phospholipids of membranes and are abundant in brain, muscle, and liver cells (14–16). Once released from the phospholipids, arachidonic acid is converted to endogenous...
bioactive eicosanoids by cyclooxygenase, lipoxygenases, and microsomal CYP2C8, CYP2C9, CYP2J, and CYP4F2 (14, 17–20). We identified three different EETs (8,9-, 11,12-, and 14,15-EETs) and 20-OH (20-HETE) in reactions catalyzed by mitochondrial WT CYP2C8 and CYP2C8 Var_3 (Fig. 7, A–D). The mitochondrial WT CYP2C8 in the presence of Adx and AdxR electron transfer proteins showed higher levels of 11,12- and 14,15-EETs than the corresponding microsomal activity driven by cytochrome P450 reductase. The mitochondrial CYP2C8 Var_3 protein, however, produced significantly lower levels of both products (Fig. 7, A and B). Notably, mitochondrial CYP2C8 Var_3 yielded a higher level of 8,9-EET and nearly a 2-fold higher level of 20-HETE than mitochondrial WT CYP2C8. The level of 20-HETE produced by mitochondrial CYP2C8 Var_3 was also more than 2-fold higher than that produced by the microsomal WT CYP2C8 reconstituted with NADPH-cytochrome P450 reductase. These results not only suggest that mitochondrial Var_3 CYP2C8 is metabolically active but also that it may be responsible for producing large amounts of 20-HETE, which is implicated to have several physiological effects (53). In all cases, montelukast inhibited the activity by 50–80%.

The catalytic activity of mitochondrially targeted Var_3 was further ascertained by reconstituting the arachidonic acid metabolism in the presence and absence of added Adx + AdxR proteins and using the CYP2C8-specific inhibitor montelukast. Fig. 7E shows that the total EET activity of Var_3-expressing mitochondria was dependent on the addition of Adx and AdxR and was inhibited by montelukast. Bacterially expressed purified Var_1 CYP2C8 also showed a high level of activity for arachidonic acid metabolism when reconstituted with Adx + AdxR (Fig. 7E).

**Roles of Var_1 (WT) CYP2C8 and CYP2C8 Var_3 in Arachidonic Acid-induced Oxidative Stress**—We used a Seahorse Bioscience metabolic flux analyzer to study the mitochondrial respiratory parameters in mock-transfected and Var_1 CYP2C8- and Var_3 CYP2C8-expressing cells. Stable cells expressing both Var_1 CYP2C8 and Var_3 proteins showed significantly lower basal respiration, maximum uncoupled respiration, and ATP-dependent respiration (Fig. 8A, panels i–iii). Treatment with 70 μM arachidonic acid had a marginal effect on all three respiratory parameters in WT CYP2C8 cells. However, arachidonic acid induced a profound 80–90% inhibition of basal, maximum uncoupled, and ATP-coupled respiration in Var_3-expressing cells. It is possible that the metabolic activity of mitochondrial Var_3 protein contributes to oxidative stress. In support of this, measurement of H_2O_2 production by the Amplex Red method (Fig. 8B) showed that Var_3-expressing cells produced the highest level of H_2O_2 in the presence of arachidonic acid. These results together support a possible role of CYP2C8 Var_3 in inducing oxidative stress.

**DISCUSSION**

CYP2C8, which is expressed in hepatic and many extrahepatic tissues, is involved in the metabolism of an array of drugs used in human medicine in addition to a proposed contribution to oxidative stress during cardiac ischemia (14–19). A number of different point mutations have been reported that affect either the gene binding ability or substrate binding (7–13). Here we report the molecular and functional characterization of some of the previously reported but not biochemically characterized splice variants of CYP2C8. Splice Var_3 (1nΔ1aΔ2a) was shown to be predominately targeted to mitochondria and shows markedly altered catalytic activity for different substrates. This appears to be the first study showing different subcellular distributions of the WT form and the N-terminal truncated Var_3 of CYP2C8 and distinctive metabolic activities of the two molecular forms of the enzyme. Analysis of human liver samples revealed that although the WT (full-length) CYP2C8 is the most abundant form expressed in the liver a faster migrating, putative 44-kDa form, characterized as Var_3, is expressed at markedly variable levels in almost all liver samples. The Var_2 form with a reading frame of 420 amino acids was detected only in some livers and at very low levels. Because of its low abundance, we did not characterize this form in terms of its catalytic activity.

The evidence for mitochondrial localization of WT and Var_3 CYP2C8 comes from multiple set experiments. First, the in vitro import of translation products into isolated mitochondria (Fig. 4) in which resistance to limited digestion with trypsin but sensitivity to Triton X-100 plus trypsin is regarded as evidence for the matrix side localization of the proteins. With this approach, we observed that ~10% of input WT CYP2C8 was imported, whereas nearly 34% of Var_3 protein was imported. The second approach involved transient transfection of COS-7 cells with cDNA constructs and quantification of mitochondrial and microsomal targeting of proteins by subcellular fractionation under minimal cross-contaminating condi-
tions followed by immunoblot analysis and by immunofluorescence co-localization of CYP2C8 with mitochondrial and microsomal markers. By all these criteria, we show that WT CYP2C8 is preferentially targeted to the ER, and a small fraction (<25%) is targeted to mitochondria. Conversely, the Var_3 protein is predominately targeted to the mitochondria and minimally associated with the ER. The ER association of this N-terminal truncated form, which lacks the transmembrane
domain, appears to be extrinsic, and other results (not presented) show that ER-associated CYP2C8 Var_3 is not catalytically active. As shown previously (27), the transmembrane organization of CYP with the N-terminal anchoring domain is important for interaction of the catalytic domain with the similarly anchored cytochrome P450 reductase.

A three-dimensional molecular model of the Var_3 protein superimposed on a CYP2C8 model (Fig. 3) shows that both the heme binding pocket and the substrate (felodipine) binding pocket are nearly intact in the truncated form. A molecular model of CYP2C8 based on x-ray crystal structure coordinates of CYP2D6, a member of the CYP2 family, and CYP2C8 (33, 36, 38, 51) shows that six Arg, His, and Tyr residues conserved in both proteins are involved in heme binding. Of these, the N-terminal Arg-97 (homologous to Arg-101 in CYP2D6) is lacking in the Var_3 protein. Despite this, the energy minimization model (Fig. 3B) suggests that the N-terminal truncated Var_3 protein is capable of binding to the heme moiety. This was further confirmed by results showing that mitochondria from stable cells expressing CYP2C8 Var_3 exhibit the characteristic CYP heme spectrum as determined by CO absorbance spectroscopy. Conversely, mitochondria from mock vector-transduced cells showed no detectable heme content. These results combined with the catalytic assays provide evidence for the heme binding ability of CYP2C8 Var_3.

Similar to CYP3A4, the substrate pocket of CYP2C8 is large but has more of a long channel rather than an open space (33, 51). This structural attribute enables both the enzymes to bind to large substrates and inhibitors, such as paclitaxel (834 Da), erythromycin (734 Da), montelukast (586 Da), etc. The N-terminal sequence stretch that forms the G and F helices is positioned over the large substrate pocket (33) and may thus facilitate the stabilization of large substrates bound on top of the enzyme. The Var_3 enzyme, which lacks the N-terminal portion of the molecule, exhibited no activity with the large substrate paclitaxel. However, the enzyme was highly active with smaller substrates, such as arachidonic acid and dibenzylfluorescein (data not shown for the latter). It is likely that the N-terminal portion of the protein forming the F and G helices is critical for the stable association of large substrates with the enzyme. Notably, the catalytic activity of Var_3 was inhibited by montelukast and proadifen, suggesting that its large substrate binding pocket is intact. These two inhibitors and a CYP2C8-specific inhibitory antibody inhibited ROS production by mitochondria from CYP2C8 Var_3-expressing cells, suggesting a possible role in the metabolism of some physiolog-

FIGURE 8. Respiratory dysfunction and ROS generation in cells treated with arachidonic acid. A, panels i–iii, the effect of arachidonic acid on respiration profile was measured using a Seahorse Bioscience XF24 extracellular flux analyzer. All parameters were analyzed using XF software and are displayed as oxygen consumption rates (pmol of O_2/min/100 µg of protein) after normalizing for the protein concentration of each well. Panel i, basal OCR accounts for baseline rates of oxygen consumption. Panel ii, 2,4-dinitrophenol-mediated uncoupling generates maximal OCR. Panel iii, inhibition by oligomycin corresponds to ATP-linked OCR. Mean values ± S.E. (error bars) were calculated based on three separate measurements. B, effects of arachidonic acid (AA) on ROS production in stable HepG2 cells. Cells were grown with or without arachidonic acid for 24 h in 6-well plates, and the culture fluids were used for assaying the levels of H_2O_2 produced using the Amplex Red method as described under "Materials and Methods." Results represent means ± S.E. (error bars) of three separate readings. * indicates a p value < 0.05, and ** indicates a p value < 0.001. ♦ in A (panel iii) indicates no detectable activity. V3, Var_3; 2C8, CYP2C8.
ical substrate(s). These results together suggest a difference in substrate preference between the intact WT CYP2C8 and its N-terminal truncated Var_3. Several studies have shown that CYP2C8 is expressed in the endothelial cells as well in the myocardium (16–20) and suggest its role in endothelial cell and myocardial toxicity. Arachidonic acid is a polyunsaturated ω-6 fatty acid produced in mammalian tissues and absorbed through dietary sources. Once released from the phospholipids by phospholipases, arachidonic acid is converted to endogenous bioactive eicosanoids by three different classes of enzymes, including CYPs belonging to CYP2C, CYP2J, and CYP4F families (11–16). The four EET regioisomers formed by epoxidation reactions are 5,6-, 8,9-, 11,12-, and 14–15-EETs. Hydroxylation activity of CYPs also produces HETEs, mainly 19-HETE and 20-HETE, of which mostly mitochondrially targeted Var_3 produced 2-fold higher levels of 20-HETE than WT CYP2C8 associated with the microsomal fraction of cells. Furthermore, arachidonic acid induced dysfunctions in stable cells expressing Var_3 protein (Figs. 7 and 8). These results further support the possibility that subcellular localization of various drug-metabolizing CYPs can have important physiological significance. Analysis of human liver samples showed widely varying levels of CYP2C8 Var_3 mRNA and proteins in different individuals. The observed variation in the level of CYP2C8 Var_3 is important because of its propensity to generate high levels of biologically active 20-HETE in different tissues. Currently, many aspects of the regulation of CYP2C8 gene expression and the regulation of differential splicing remain unclear. However, this study demonstrates the importance of transcriptionally targeted CYPs and their roles in drug metabolism and toxicity.

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REFERENCES
1. Rendic, S., and Di Carlo, F. J. (1997) Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers and inhibitors. Drug Metab. Rev. 29, 413–580
2. Enayetallah, A. E., French, R. A., Thibodeau, M. S., and Grant, D. F. (2004) Distribution of soluble epoxide hydrolase and of cytochrome P450 2C8, 2C9, and 2J2 in human tissues. J. Histochem. Cytochem. 52, 447–454
3. Klose, T. S., Blaisdell, J. A., and Goldstein, J. A. (1999) Gene structure of CYP2C8 and extrahepatic distribution of the human CYP2Cs. J. Biochem. Mol. Toxicol. 13, 289–295
4. Thum, T., and Borlak, J. (2000) Gene expression in distinct regions of the heart. Lancet 355, 979–983
5. Nishimura, M., Yaguti, H., Yoshitsugu, H., Naito, S., and Satoh, T. (2003) Tissue distribution of mRNA expression levels of human cytochrome P450 isoforms assessed by high selectivity real-time reverse transcription PCR. Yakugaku Zasshi 123, 369–375
6. Knüfer, H., Schmidt, R., Stainitz, D., Brauckhoff, M., Schönfelder, M., and Preiss, R. (2004) CYP2C19 and IL-6 expression in breast cancer. Breast 13, 28–34
7. Rahman, A., Korzekwa, K. R., Grojan, J., Gonzalez, F. J., and Harris, I. W. (1994) Selective biotransformation of Taxol to 6α-hydroxytaxol by human cytochrome P450 2C8. Cancer Res. 54, 5543–5546
8. Daily, E. B., and Aquilante, C. L. (2009) Cytochrome P450 2C8 pharmacogenetics: a review of clinical studies. Pharmacogenomics 10, 1489–1510
9. Totah, R. A., and Rettie, A. E. (2005) Characteristics of CYP2C8 and human CYP2C family members. Clin. Pharmacol. Ther. 77, 341–352
10. VandenBrink, B. M., Foti, R. S., Rock, D. A., Wiencek, L. C., and Wahlstrom, J. L. (2011) Evaluation of CYP2C8 inhibition in vitro: utility of montelukast as a selective CYP2C8 probe substrate. Drug Metab. Dispos. 39, 1546–1554
11. Bahadur, N., Leathart, J. B., Mutch, E., Steimel-Crespi, D., Dunn, S. A., Gilissen, R., Houdt, J. V., Hendrickx, J., Mannens, G., Bobets, H., Williams, F. M., Armstrong, M., Crespi, C. L., and Daly, A. K. (2002) CYP2C8 polymorphisms in Caucasians and their relationship with paclitaxel 6α-hydroxylation activity in human liver microsomes. Biochem. Pharmacol. 64, 1579–1589
12. Lundblad, M. S., Stark, K., Eliasson, O., Olow, E., and Rane, A. (2005) Biosynthesis of epoxyeicosatrienoic acids varies between polymorphic CYP2C enzymes. Biochem. Biophys. Res. Commun. 327, 1052–1057
13. Yasar, U., Bennet, A. M., Eliasson, E., Lundgren, S., Wiman, B., and De Faire, U., and Rane, A. (2003) Allelic variants of cytochromes P450 447 and 454 modify the risk for acute myocardial infarction. Pharmacogenetics 13, 715–720
14. Roman, R. J. (2002) P450 metabolites of arachidonic acid in the control of cardiovascular function. Physiol. Rev. 82, 131–185
15. Xu, X., Zhang, X. A., and Wang, D. W. (2011) The role of CYP450 epoxygenase and metabolites, epoxyeicosatrienoic acids in cardiovascular and malignant diseases. Adv. Drug Deliv. Rev. 63, 597–609
16. Zeldin, D. C. (2001) Epoxygenase pathways of arachidonic acid metabolism. J. Biol. Chem. 276, 36059–36062
17. Edin, M. L., Wang, Z., Bradbury, J. A., Graves, J. P., Lih, F. B., DeGraff, L. M., Foley, J. F., Torphy, R., Ronnekleiv, O. K., Tomer, K. B., Lee, C. R., and Zeldin, D. C. (2011) Endothelial expression of human cytochrome P450 epoxygenase CYP2C8 increases susceptibility to ischemia-reperfusion injury in isolated mouse heart. FASEB J. 25, 3436–3447
18. Wu, S., Chen, W., Murphy, E., Gabel, S., Tomer, K. B., Foley, J., Steenbergen, C., Falc, J. R., Moomaw, C. R., and Zeldin, D. C. (1997) Molecular cloning, expression and functional significance of a cytochrome P450 highly expressed in rat heart myocytes. J. Biol. Chem. 272, 12551–12559
19. Delozier, T. C., Kissling, G. E., Coulter, S. J., Dai, D., Foley, J. F., Bradbury, J. A., Murphy, E., Steenbergen, C., Zeldin, D. C., and Goldstein, J. A. (2007) Detection of human liver CYP2C8, CYP2C9, and CYP2J2 in cardiovascular tissues. Drug Metab. Dispos. 35, 682–688
20. Granville, D. J., Tashakkor, B., Takeuchi, C., Gustafsson, A. B., Huang, C., Sayen, M. R., Wentworth, P. Jr., Yeager, M., and Gottlieb, R. A. (2004) Reduction of ischemia and reperfusion-induced myocardial damage by cytochrome P450 inhibitors. Proc. Natl. Acad. Sci. U.S.A. 101, 1321–1326
21. Avadhani, N. G., Sangar, M. C., Bansal, S., and Bajpai, P. (2011) Bimodal targeting of cytochrome P450s to endoplasmic reticulum and mitochondria: the concept of chimeric signals. FEBS J. 278, 4218–4229
22. Neve, E. P., and Ingelman-Sundberg, M. (2008) Intracellular transport and localization of microsomal cytochrome P450. Anal. Bioanal. Chem. 392, 1075–1084
23. Omura, T. (2006) Mitochondrial P450s. Chem.-Biol. Interact. 163, 86–93
24. Addya, S., Anandatheerthavarada, H. K., Biswas, G., Bhagwat, S. V., Mullik, J., and Avadhani, N. G. (1997) Targeting of NH2-terminal processed microsomal protein to mitochondria: a novel pathway for the biogenesis of hepatic mitochondrial P450MT2. J. Cell Biol. 139, 589–599

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25. Anandatheerthavarada, H. K., Addya, S., Dwivedi, R. S., Biswas, G., Mullick, I., and Avadhani, N. G. (1997) Localization of multiple forms of inducible cytochrome P450 in rat liver mitochondria: immunological characteristics and patterns of xenobiotic substrate metabolism. Arch. Biochem. Biophys. 339, 136–150

26. Anandatheerthavarada, H. K., Biswas, G., Mulllick, J., Sepuri, N. B., Otvos, L., Pain, D., and Avadhani, N. G. (1999) Dual targeting of cytochrome P4502B1 to endoplasmic reticulum and mitochondria involves a novel signal activation by cyclic AMP-dependent phosphorylation at Ser128. EMBO J. 18, 5494–5504

27. Bhagwat, S. V., Biswas, G., Anandatheerthavarada, H. K., Addya, S., Pan- dak, V., and Avadhani, N. G. (1999) Dual targeting property of the N-terminal signal sequence of P450 1A1. Targeting of heterologous proteins to endoplasmic reticulum and mitochondria. J. Biol. Chem. 274, 24014–24022

28. Robin, M. A., Anandatheerthavarada, H. K., Biswas, G., Sepuri, N. B., Gordon, D. M., Pain D., and Avadhani, N. G. (2002) Bimodal targeting of microsomal CYP2E1 to mitochondria through activation of an N-terminal chimeric signal by cAMP mediated phosphorylation. J. Biol. Chem. 277, 40583–40593

29. Sangar, M. C., Anandatheerthavarada, H. K., Tang, W., Prabu, S. K., Martin, M. V., Dostalek, M., Guengerich, F. P., and Avadhani, N. G. (2009) Human liver mitochondrial CYP2D6: individual variations and implications in drug metabolism. FEBS J. 276, 3440–3453

30. Bansal, S., Anandatheerthavarada, H. K., Prabu, G. K., Milne, G. L., Martin, M. V., Guengerich, F. P., and Avadhani, N. G. (2013) Human cytochrome P450 2E1 mutations that alter mitochondrial targeting efficiency and susceptibility to ethanol-induced toxicity in cellular models. J. Biol. Chem. 288, 12627–12644

31. Boopathi, E., Anandatheerthavarada, H. K., Bhagwat, S. V., Biswas, G., Fang, J. K., and Avadhani, N. G. (2000) Accumulation of mitochondrial CYP2MT2, NH2-terminal truncated cytochrome P4501A1 in rat brain during chronic treatment with β-naphthoflavone. A role in the metabolism of neuroactive drugs. J. Biol. Chem. 275, 34415–34423

32. Bansal, S., Lee, A. N., Gonzalez, F. J., Guengerich, F. P., Chowdhury, A. R., Anandatheerthavarada, H. K., and Avadhani, N. G. (2014) Mitochondrial targeting of cytochrome P450 (CYP) 1B1 and its role in polyyclic aromatic hydrocarbon-induced mitochondrial dysfunction. J. Biol. Chem. 289, 9936–9951

33. Johnson, E. F., and Stout, C. D. (2008) Determinants of cytochrome P450 2C8 substrate binding. J. Biol. Chem. 283, 17227–17237

34. Klein, K., Tatzel, S., Sepuri, N. B., Otvos, L., Pain, D., and Avadhani, N. G. (1999) Dual targeting of cytochrome P4502B1 to endoplasmic reticulum and mitochondria involves a novel signal activation by cyclic AMP-dependent phosphorylation at Ser128. EMBO J. 18, 5494–5504

35. Tang, Z., Martin, M. V., and Guengerich, F. P. (2009) Elucidation of functions of human cytochrome P450 enzymes: identification of endogenous substrates in tissue extracts using metabolic and isotopic labeling approaches. Anal. Chem. 81, 3071–3078

36. Schoch, G. A., Yano, J. K., Wester, M. R., Griffin, K. J., Stout, C. D., and Johnson, E. F. (2004) Structure of human microsomal P450 2C8. Evidence for a peripheral fatty acid binding site. J. Biol. Chem. 279, 9497–9503

37.ᶲ 289, 20619–20627

38. Williams, P. A., Cosme, J., Sridhar, V., Johnson, E. F., and McRee, D. E. (2000) Mammalian cytochrome P450 monoxygenase: structural adaptations for membrane binding and functional diversity. Mol. Cell 5, 121–131

39. Ryan, M. T., Müller, H., and Pfanner, N. (1999) Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. Am. J. Physiol. Cell Physiol. 292, C125–C136

40. Ishizuka, T., Cheng, J., Singh, H., Falck, J. R., Abraham, N. G., Shapiro, J., and Schwartzman, M. L. (2004) Vascular cytochrome P450 4A expression and caveolae-dependent protein kinase C-mediated protein phosphorylation modulates cytochrome c oxidase function and augments hypoxia and myocardial ischemia related injury. J. Biol. Chem. 281, 2061–2070

41. Wu, M., Neilson, A., Swift, A. L., Moran, R., Tamagnine, J., Parslow, D., Armstead, S., Lemiure, K., Orrell, J., Teich, J., Chomicz, S., and Ferrick, D. A. (2007) Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. Am. J. Physiol. Cell Physiol. 292, C125–C136

42. Ishizuka, T., Cheng, J., Singh, H., Falck, J. R., Abraham, N. G., Shapiro, J., and Schwartzman, M. L. (2004) Vascular cytochrome P450 4A expression and caveolae-dependent protein kinase C-mediated protein phosphorylation modulates cytochrome c oxidase function and augments hypoxia and myocardial ischemia related injury. J. Biol. Chem. 281, 2061–2070

43. Singh, H., Cheng, J., Dang, H., Kemp, R., Ishizuka, T., Nasjletti, A., and Schwartzman, M. L. (2007) Vascular cytochrome P450 4A expression and 20-hydroxyeicosaatetraenoic acid stimulates nuclear factor-κB activation and the production of inflammatory cytokines in human endothelial cells. J. Pharmacol. Exp. Ther. 324, 103–110

44. Singh, H., Cheng, J., Dang, H., Kemp, R., Ishizuka, T., Nasjletti, A., and Schwartzman, M. L. (2007) Vascular cytochrome P450 4A expression and 20-hydroxyeicosatetraenoic acid stimulates nuclear factor-κB activation and the production of inflammatory cytokines in human endothelial cells. J. Pharmacol. Exp. Ther. 324, 103–110

45. Chen, P., Guo, M., Wygle, D., Edwards, P. A., Falck, J. R., Roman, R. J., and Scioli, A. G. (2005) Inhibitors of cytochrome P450 4A suppress angiogenic responses. Am. J. Pathol. 166, 615–624